University of Glasgow

Investigation of pertussis toxin A- and B-subunit activities in acellular vaccines by enzymatic and carbohydrate-binding assays

Sheena Robin Gomez

B.Sc. Hons

A thesis submitted in partial fulfillment of the requirement for the Degree of Doctor of Philosophy

Division of Bacteriology
National Institute for Biological Standards and Control

Division of Infection and Immunity
Institute of Biomedical and Life Sciences
University of Glasgow

January 2007

Dedication

For my father, the late Kochupappu Ramakrishnan, my mother,
Vasanthakumari Ramakrishnan and my husband, Robin Gomez
'A life without cause is a life without effect'

Acknowledgements

First and foremost I wish to acknowledge the two key people that have played a vital role for the completion of this thesis: Doctors Chun-Ting Yuen and Dorothy Xing. I wish to thank them both for their guidance, good teaching, critical comments, and for their continual encouragement and kindness throughout the time it took me to complete this research and write the thesis. I would have been lost without them. I would also like to thank Alan Dalan Wang for the significant contribution he made with the final stages of this project with reference to the mathematical model. Special thanks also go to Dr Jun Wheeler. Your constant enthusiasm and passion for the subject was really inspiring and made it a pleasure to work with you. I cannot overstate my gratitude for teaching me proteomics and for the fruitful discussions. I would also like to thank Doctors Michael Corbel, John Coote and Roger Parton for their support and most valuable comments throughout the years they have reviewed my work. Although I cannot name them all, I would like to sincerely thank my colleagues/friends: Alex, Cathy, Gordon, Louise, Michael, Noela, Penny, Peter, Sandra, Satnam, Steve, Tom, Yvonne, Pub and uni lot with special reference to Nutan and Sarah. Your support and contribution in many different ways were essential for the completion of this thesis. I wish to thank my entire family for their unconditional love and support: My husband, my late father, mother, mother-in-law, brothers, sister-in-laws, nephews and nieces; thank you! Last of all I would like to thank the Home Office for providing the funds to carry out this work

'When you get to the end of your rope, tie a knot and hang on'
Franklin D. Roosevelt

Abstract

Pertussis toxin (PT) is a major virulence factor produced by *Bordetella pertussis*. In its detoxified form (PTd), it is an important component of acellular pertussis vaccines although some residual PT activity may be present because of the limitations of the detoxification processes used. The *in vivo* histamine sensitisation test (HIST) in mice is currently used for the safety testing of these vaccines to determine the level of their residual PT activity. However, an alternative test is needed because of large assay variability and ethical concerns with regard to animal usage. The main objective of this study was to search for an alternative test to the HIST.

The ADP-ribosylation enzyme activity of PT is thought to be the major factor responsible for the histamine-sensitising activity detected *in vivo*. In the present study, the enzymatic activities in different acellular pertussis-based combination vaccine formulations were measured by a recently-developed ADP-ribosylation assay and compared with their reactivities in the HIST. The results indicated that different products showed differences in ADP-ribosylation activity and, these did not correlate with their reactivity in the HIST.

PT has two functionally-distinct domains: the enzymatic A-protomer and the B-oligomer that facilitates host-cell binding and entry of PT into the cell. This dual biological function could explain why the residual enzyme activity of PT in vaccines did not fully reflect the *in vivo* reactivity observed by the HIST. Thus, refinement of the *in vitro* test to include a step which monitored the B-subunit activity of PT was attempted. A quantitative PT carbohydrate-binding assay using glycoproteins or defined

oligosaccharides was developed. PT was found to bind preferentially to multiantennary *N*-glycans, with the highest binding towards the fully sialylated structures. In contrast, PTd lost the ability to bind to sialylated multiantennary structures but retained some capacity to bind to neutral multiantennary structures.

Different vaccine preparations had different levels of PT binding activity as well as enzymatic activity. It was concluded that, although the enzymatic activity of PT plays a more important role in the death of mice in the HIST, a high binding activity of the B-subunit could increase the *in vivo* toxic effect by aiding the accessibility of the A-subunit to its cellular targets. A mathematical equation was devised to establish a preliminary relationship between the enzymatic, carbohydrate-binding and HIST assays in a product-dependent manner. Further studies with a larger number of vaccines are required for a more meaningful statistical analysis. However the methods form a sound basis for the future development of an alternative assay to the histamine challenge test. The *in vitro* assays could also be useful for investigating the mechanisms of PT detoxification. Comparisons of A- and B-subunit activities of purified PT and vaccine preparations of PTd indicated that both subunits are modified after chemical detoxification. Different vaccine products had different levels of enzymatic and binding activities and it was concluded that different detoxification procedures, as well as formulation factors, could contribute to this variation.

A CHO cell clustering assay is used as an alternative *in vitro* test to the HIST for assessing residual PT activity at the bulk stage of vaccine production. In a parallel study to the above, comparative proteomics was used to gain insights into the mechanism of PT-induced CHO cell clustering with a view to developing a mechanistic-based

alternative assay for the safety testing of pertussis-based combination vaccines. A proteomic map of CHO cells was established and PT-induced CHO cell clustering appeared to be a complex process involving subtle changes in various cellular functions, mainly related to intracellular transport, cell stress and the cell cycle. The information obtained will be useful for future studies into the possible mechanisms of the effect of PT on CHO cells.

Abbreviations

A1 = mono sialylated biantennary
A2 = di-sialylated biantennary
A2-1 = di-sialylated (1 Ngc, 1NAc) biantennary
A2-2 = di-sialylated (2Ngc) biantennary
A2-3 = tri-sialylated (1Ngc, 2NAc) biantennary
A2-4 = tri-sialylated (3Ngc) biantennary
A3 = tri-sialylated triantennary
A3-1 = mixture of tri-sialylated (1Ngc, 2NAc) and tri-sialylated (2Ngc, 1NAc)
A3F = tri-sialylated triantennary with core fucose
A4 = tetra-sialylated tetraantennary
A4F = tetra-sialylated tetraantennary with core fucose
ACT = adenylate cyclase toxin
ACV = acellular pertussis vaccines
Afet = asialofetuin
ATP = adenosine triphosphate
β-NAD = β-nicotinamide adenine dinucleotide
Biotin = 3-aminopropylbiotinamide
bAGP = bovine α_1-acid glycoprotein
bTG = bovine thyroglobulin G
BTN = biotinylated
BrkA = *Bordetella* resistance to killing
BSA = bovine serum albumin
^{0}C = degrees Celsius
CHO cells = Chinese hamster ovary cells
D = diphtheria toxoid
DAB = 3'-diaminobenzidine
DHPE = 2-dihexadecyl-sn-glycero-3phosphoethanolamine
DNT = dermonecrotic toxin
DTaP= diphtheria tetanus acellular pertussis vaccine
DTPw= diphtheria tetanus whole cell pertussis vaccine
DTT = dithiothreitol
DMSO = dimethyl sulphoxide
EDTA = ethylenediaminetetraacetic acid
ELISA = enzyme-linked immunosorbent assay
FA = formaldehyde
FAC TAG = fluorescein tag
FCS = foetal calf serum
Fet = fetuin
FHA = filamentous haemagglutinin
Fim = fimbriae
Fims2/3 = fimbriae 2 and 3
Gal = galactose
GA = glutaraldehyde
Glc= glucose
g/mg/μg/ng = grams/milligrams/micrograms/nanograms
h = hours
HB = hepatitis B
HCl = hydrochloric acid

HIST = histamine sensitisation test
hAGP = human α_1-acid glycoprotein
hHg = human haptoglobin
hTf = human transferrin
IEF = isoelectric focusing
IPG = Immobiline pH gradient
i.p = intraperitoneal
i.v = intravenous
IPV = inactivated polio virus
ISP = isopropanol
IU = international units
Lac = lactose
l/ml/µl = litres/millilitres/microlitres
LNT = lacto-N-tetraose
LNnT = lacto-N-neotetraose
LNFP-I = lacto-N-fucopentaose-I
LNFP-II = lacto-N- fucopentaose-II
LNFP-III = lacto-N-fucopentaose-III
LPC = lysophosphatidylcholine
LPS = lipopolysaccharide
MALDI-TOF = matrix assisted laser desorption ionisation-time of flight
Maltopentaose = Glc5
M/mM/µM/nM/pM = Molar/millimolar/micromolar/nanomolar/ picomolar
m/nm/µm = metre/nanometre/micrometre
MeOH = methanol
min = minutes
MS = mass spectrometry
MWGT = mouse weight gain test
NA2 = asialo-biantennary
NA3 = asialo-triantennary
NA4 = asialo-tetraantennary
NAc = *N*-acetyl
Ngc = *N*-glycolyl
NO = nitric oxide
OD= optical density
O/N = overnight
OPD = *o*-phenylenediamine dihydrochloride
OVA = ovalbumin
PAGE = polyacrylamide gel electrophoresis
PBS = phosphate buffered saline
PBSG = PBS containing gelatin
PBST = PBS containing Tween-20
PMSF = phenylmethylsulphonyl fluoride
PRN = pertactin
PRP = polyribosylribitol phosphate from *haemophilus influenzae* type b
PRP-T or PRP-D = PRP conjugated to tetanus or diphtheria toxoid
PT = pertussis toxin
PTd = chemically detoxified PT
PT-g = genetically inactivated PT
RNaseA = ribonuclease A

RNaseB = ribonuclease B
RT = room temperature
SaLac = sialyl α2-3/6-lactose
Sa3LNT = sialyl α2,3-LNT
Sa3LNnT = sialyl α2,3-LNnT
Sa3LeA5 = sialyl LNFP-II
SDS = sodium dodecyl sulphate
SHD = single human dose
Su3LNT = 3'-sulphated LNT
Su3LeX5 = 3'-sulphated LNFP-III
Su3LeA5 = sulphated LNFP-II
$TBACNBH_3$ = tetrabutylammonium cyanoborohydride
T = tetanus toxoid
TCF = tracheal colonisation factor
TCT = tracheal cytotoxin
TFA = trifluoroacetic acid
Tween-20 = polyoxyethylene 20 sorbitan monolaurate
Tween-80= polyoxyethylene 80 sorbitan monolaurate
WCV= whole cell pertussis vaccines

List of Contents

Title	i
Acknowledgements	iii
Abstract	iv
Abbreviations	vii
List of contents	x
List of figures	xiv
List of tables	xv
Publications and poster communications	xvi
Chapter 1: Introduction	1
1.1 The pathogen, the disease and treatment	2
1.2 Epidemiology and prevention of pertussis	5
1.3 Pathogenicity of *B. pertussis*	6
1.3.1 Regulation of virulence factors	6
1.3.2 Adhesins	8
1.3.2.1 Filamentous haemagglutinin (FHA)	8
1.3.2.2 Fimbriae (Fim)	9
1.3.2.3 Pertactin (PRN), tracheal colonisation factor A (tcfA) and *Bordetella* resistance to killing protein (BrkA)	11
1.3.3 Toxins	12
1.3.3.1 Adenylate cyclase toxin (ACT)	12
1.3.3.2 Demonecrotic toxin (DNT)	13
1.3.3.3 Lipopolysaccharide (LPS)	14
1.3.3.4 Tracheal cytotoxin (TCT)	14
1.3.3.5 Pertussis toxin (PT)	15
1.4 PT	15
1.4.1 Structure and biogenesis	15
1.4.2 Molecular mechanisms of toxin action	16
1.4.2.1 Binding	18
1.4.2.2 Entry	20
1.4.2.3 ADP-ribosyl transferase activity	21
1.4.3 Biological activities of PT	24
1.4.3.1 Lymphocytosis promoting activity	24
1.4.3.2 Hyperinsulinaemia with subsequent hypoglycaemia	25
1.4.3.3 Histamine sensitisation activity	26
1.4.3.4 Clustering of CHO cells	27
1.4.3.5 T-cell mitogenicity	28
1.4.3.6 Other activities of PT	29
1.4.4 Detoxification of PT	31
1.5 Pertussis vaccines	34
1.5.1 Whole-cell pertussis vaccines (WCVs)	34
1.5.2 Acellular pertussis vaccines (ACVs)	35
1.5.2.1 Acellular-based combination vaccines	38
1.6 Quality control testing of pertussis vaccines	41
1.6.1 Potency and toxicity tests for WCVs	42
1.6.1.1 Mouse weight gain test	42
1.6.1.2 Intracerebral mouse protection test	43
1.6.1.3 Alternative potency tests	44

1.6.2 Potency and toxicity tests for ACV 47
1.6.2.1 Specific toxicity tests 47
1.6.2.2 Alternative specific toxicity test 49
1.6.2.3 Potency test 51
1.7 Aims and objectives 52

Chapter 2: Materials and Methods 54
2.1 Antigens 55
2.1.1 PT 55
2.1.2 Pertussis toxoid 55
2.1.3 Other antigens 55
2.2 Antisera 56
2.3 Test vaccines 56
2.4 Determination of protein concentration 56
2.4.1 UV absorption spectroscopy 56
2.4.2 Modified Bradford assay 58
2.5 Histamine sensitisation test (HIST) 59
2.6 HPLC 59
2.7 ADP-ribosylation assay 60
2.7.1 Enzymatic reaction 60
2.7.2 Analysis of ADP-ribosylated product and FAC-$G_{\alpha i3}$C20 by reverse-phase HPLC 62
2.7.3 Data analysis 64
2.8 Development of a carbohydrate-binding assay for the B-oligomer of pertussis toxin and toxoid 64
2.8.1 General materials 64
2.8.2 Glycoproteins, glycolipids and neoglycolipids 65
2.8.3 Oligosaccharides 68
2.8.4 Preparation of biotinylated (BTN) glycans and glycoproteins 68
2.8.4.1 Conjugation of oligosaccharides or glycoproteins to biotin 68
2.8.4.2 Purification of biotinylated oligosaccharides by normal phase HPLC 72
2.8.4.3 Identification of biotinylated oligosaccharides 73
2.8.4.4 Quantification of biotinylated oligosaccharides by orcinol staining 74
2.8.5 Investigation of PT binding to glycolipids using DOT blot method 75
2.8.6 Investigation of PT binding to neoglycolipids by neoglycolipid-capture ELISA 75
2.8.7 Screening of glycoproteins as ligands for PT binding 76
2.8.8 Screening of oligosaccharides as ligands for PT binding 76
2.8.9 Determination of binding activity of PT and PTd using glycoprotein- or oligosaccharide-capture ELISA 77
2.8.10 Desorption of antigens from aluminium adjuvant suspensions 78
2.9 Exploring the potential applications of ADP-ribosylation assay and carbohydrate-binding assay 79
2.9.1 Comparison of different formulations 79
2.9.2 Preliminary interpretation of the relationship between the *in vitro* and *in vivo* tests 80
2.10 Proteomic analysis of PT-induced CHO cell clustering 81
2.10.1 Preparation of CHO cells 81

2.10.1.1 Cell culture 81
2.10.1.2 Treatment of CHO cells with PT 82
2.10.1.3 Sample preparation for gel electrophoresis 82
2.10.2 Two dimensional (2-D) gel electrophoresis 83
2.10.2.1 Isoelectric focusing (IEF) 84
2.10.2.2 IPG strip equilibration 84
2.10.2.3 Second dimensional gel electrophoresis 84
2.10.3 Protein staining 85
2.10.4 Gel analysis using computer assisted 2-D software programme 85
2.10.5 Enzymatic digestion and mass spectrometry for protein identification 86

Chapter 3 Results 88
3.1 ADP-ribosylation assay: Standardisation and assessment of residual PT ADP-ribosyl transferase activity in pertussis vaccines and its relationship with the *in vivo* HIST 89
3.1.1 Selection of substrate for ADP-ribosylation assay 89
3.1.1.1 Characterisation of FAC-$(CH_2)_5$-$G_{\alpha i3}$C20 and FAC-$G_{\alpha i3}$C20 peptide substrates 90
3.1.1.2 Comparison of FAC-$(CH_2)_5$-$G_{\alpha i3}$C20 and FAC-$G_{\alpha i3}$C20 as peptide substrates for PT 92
3.1.2 Standardisation of ADP-ribosylation assay 92
3.1.3 Residual PT ADP-ribosylation activities in different pertussis vaccine formulations 98
3.1.4 Comparison of residual PT ADP-ribosylation activities and their relationship with the *in vivo* HIST 98
3.2 Development of a carbohydrate-binding assay for pertussis toxin and toxoid 103
3.2.1 Characterisation of biotinylated oligosaccharides 103
3.2.1 Screening of glycoproteins and oligosaccharides as ligands for PT binding using ELISA 107
3.2.3 Screening of glycolipids as ligands for PT 110
3.2.4 Comparison of PT and PTd binding in glycoconjugate-capture ELISA 111
3.2.5 Effect of other vaccine components on the binding assay 111
3.2.6 De-sorption of antigens from aluminium adjuvant solution 114
3.2.7 Validation of carbohydrate-binding assay using A3-capture ELISA 118
3.3 Exploring the potential application of ADP-ribosylation assay and carbohydrate-binding assay 120
3.3.1 Comparison of enzymatic and binding activities of PT in vaccine formulations and in purified native PT 120
3.3.2 Comparison of different detoxification methods 121
3.3.3 Comparison of different formulations 121
3.3.2.1 Preliminary investigation of the effect of Tween-80 on enzymatic and binding activities of vaccines 127
3.3.4 Relationship of activities of A- and B- subunits of PT/d in vaccines and the *in vivo* HIST 132
3.3.4.1 Role of A- and B- subunit activities in vaccines and in the HIST 132
3.3.4.2 Preliminary interpretation of the relationship between *in vitro* and *in vivo* tests of PT toxicity 134

Chapter 4 Discussion 144
4.1 Standardisation and assessment of residual PT ADP-ribosyl transferase

activity in pertussis vaccines and its relationship with the *in vivo* HIST 145
4.1.1 Optimisation and standardisation of the ADP-ribosylation assay 145
4.1.2 Residual PT ADP-ribosylation activities in different pertussis vaccine formulations 147
4.1.3 Relationship between residual PT activity in vaccines and reactivity in the HIST 149
4.2 Development of a carbohydrate-binding assay for pertussis toxin and toxoid 153
4.2.1 Carbohydrate-binding specificity of PT 153
4.2.2 Binding of PT to glycolipids 156
4.2.3 Comparison of PT and PTd binding in glycoconjugate-capture ELISA 157
4.2.4 Validation of carbohydrate-binding assay using A3-capture ELISA 159
4.3 Exploring the potential application of the ADP-ribosylation and carbohydrate-binding assays 161
4.3.1 Differential modification of A- and B-subunit activities of PT after chemical detoxification 161
4.3.2 Comparison of different detoxification procedures 162
4.3.3 Comparison of different formulations 163
4.3.4 Relationship between A- and B- subunit activities of PT and the *in vivo* HIST 165
4.3.4.1 Comparison of A- and B-subunit activities of PT in vaccines and in purified PT 90/518 and their relationship with HIST 165
4.3.4.2 Role of A- and B-subunit activities in vaccines and the *in vivo* HIST 167
4.3.5 Establishment of a preliminary relationship between enzymatic, carbohydrate-binding and HIST tests of PT toxicity 168

Chapter 5 Proteomic analysis of PT-induced CHO cell clustering 170
5.1 Introduction 171
5.2 Results 175
5.2.1 Determination of optimal sampling conditions and IEF times for the proteomic profiling of CHO cells 175
5.2.2 2-D PAGE using narrow range IPG strips 177
5.2.3 Comparison of control and toxin-treated CHO cell protein expression profiles 179
5.2.4 Protein identification 183
5.3 Discussion 188
5.3.1 Proteome map of CHO cells 188
5.3.2 Possible roles of differentially expressed proteins in CHO cells treated with PT 189
5.3.3 Technical considerations 194
Appendices 196
Appendix A HIST solutions 196
Appendix B ADP-ribosylation assay solutions 196
Appendix C Quantification of BTN-oligosaccharide solution 198
Appendix D 2D-PAGE 198
Appendix E Experimental data 201
Appendix F Acknowledgement letter from Home Office for research conducted 214
Appendix G Publication 214
Bibliography 226

List of Figures

Fig 1.1	Attachment of *B. pertussis* to the ciliated epithelium of the respiratory tract	4
Fig 1.2	Typical virulence factors associated with the pathogenesis of pertussis	7
Fig 1.3	Schematic picture of PT	17
Fig 1.4	Schematic representation of G-protein-coupled receptor signalling and the mechanism of PT action	23
Fig 2.1	Structure of fluorescein tag (FAC TAG) a) with and b) without linker	61
Fig 2.2	Reaction scheme of PT-catalysed ADP-ribosylation of FAC TAG-$G_{i\alpha 3}$ C20 peptide	63
Fig 2.3	Reaction scheme for formation of biotinylated oligosaccharides.	71
Fig 3.1	HPLC of FAC-$G_{\alpha i3}$C20 substrate	91
Fig 3.2	Typical chromatograms showing the product of the PT-catalysed ADP-ribosylation of FAC-$G_{\alpha i3}$C20	91
Fig 3.3	Comparison of FAC-$G_{\alpha i3}$C20 (A) and FAC-$(CH_2)_5$-$G_{\alpha i3}$C20 (B) peptide substrates as substrates for PT-catalysed ADP-ribosylation reaction	93
Fig 3.4	Comparison of dose-response curves for ADP-ribosylation by PT in the presence of adjuvants	95
Fig 3.5	ADP-ribosylation activities in different pertussis vaccine formulations and relationship to activities in the in vivo HIST	100
Fig 3.6	Comparison of ADP-ribosylation activities of paired DTaP-C and DTaP-D products	102
Fig 3.7	Typical Amide-80 HPLC chromatograms of A3-BTN and NA3-BTN after partial purification by ethyl acetate extraction	104
Fig 3.8	Binding of PT to glycoproteins	108
Fig 3.9	(a) Binding of PT to glycoproteins and (b) Binding of PT to subgroups of BTN-*N*-glycans	109
Fig 3.10	Binding of PT to (neo)glycolipids	112
Fig 3.11	Binding of PT and PTd to oligosaccharides A3 and NA3 using oligosaccharide-ELISA	113
Fig 3.12	Binding activity of PT in the presence of other vaccine components.	115
Fig 3.13	Effect of desorption reagents on ADP-ribosylation activity in DTaP preparations	116
Fig 3.14	Fetuin binding activity of PTd eluted from $Al(OH)_3$ and $AlPO_4$ adsorbed vaccine preparations (DTaP, acellular pertussis vaccine preparation) using different de-sorption agents	117
Fig 3.15	ADP-ribosylation (a) and A3 binding (b) activities of PT in vaccines compared to purified PT	122
Fig 3.16	HIST results for DTaP-D (x) and corresponding batches of DTaP-C	123
Fig 3.17	Comparison of the (a) ADP-ribosylation and (b) binding activities of PT present in DTaP-D (x) and their corresponding batches of DTaP-C vaccine products	125
Fig 3.18	Comparison of ratio of PT-B activity to PT-A activity in the vaccines.	126
Fig 3.19	Effect of concentrations of Tween-80 on DTaP-C ADP-ribosylation activity	128
Fig 3.20	Time course of Tween effect on DTaP-C (x)	128
Fig 3.21	Effect of Tween-80 on different vaccine products	130

Fig 3.22	Comparison of the binding of monoclonal antibodies to S1 and S2/3 to DTaP-A and DTaP-C products in the presence and absence of 0.1% Tween-80	131
Fig 3.23	Comparison of PT A- and B- subunit activities and the *in vivo* toxic activity using HIST in DTaP-D, E and F vaccines	133
Fig 3.24	A- x B-subunit activities in pertussis vaccines	136
Fig 3.25	Relationship between enzymatic, binding and HIST activities for DTaP-A (n=9)	140
Fig 3.26	Relationship between enzymatic, binding and HIST activities for DTaP-C (n= 6)	141
Fig 3.27	Relationship between enzymatic, binding and HIST activities for DTaP-D (n=14).	142
Fig 5.1	The effect of different solubilising methods and IEF times on protein separation and resolution in 2D-PAGE	176
Fig 5.2	The effect of different IEF times on protein separation and resolution in 2D-PAGE using pH 6-9 NL IPG strips	178
Fig 5.3	PT induced clustering effect on CHO cells	180
Fig 5.4	2D-PAGE protein profiles of the control and PT-treated CHO cell extracts	182
Fig 5.5	2D-PAGE proteome map of CHO cell proteins	184

List of Tables

Table 1.1	Reactivities of the different chemical reagents used to inactivate PT	33
Table 1.2	Existing and potential acellular pertussis-based combination vaccines	39
Table 2.1	Pertussis vaccines	57
Table 2.2	Structures of glycolipids and neoglycolipids	66
Table 2.3	*N*-glycan structures predominantly found on glycoproteins	67
Table 2.4	Structures of neutral oligosaccharides	69
Table 2.5	Structures of acidic oligosaccharides	70
Table 3.1	Assay characteristics for ADP-ribosylation assay	97
Table 3.2	ADP-ribosylation activities in different pertussis vaccine formulations	99
Table 3.3	Monosaccharide compositions, approximate hexose contents and mass spectrometry data of BTN-glycan structures	105
Table 3.4	Assay reproducibility and validation for A3-capture ELISA for PT eluted from different DTaP preparations by tri-sodium citrate	119
Table 3.5	Comparison of *in vitro* and *in vivo* activities for different pertussis-based combination vaccines	135
Table 3.6	Comparison of actual deaths due to purified PT preparation in HIST and predicted deaths from enzymatic and binding data using equation 4	138
Table 5.1	Number of spots identified and matched to reference image	181
Table 5.2	Proteins identified from CHO cell extracts	185
Table 5.3	Protein functions of identified proteins from CHO cells treated with PT	187

Publications and poster communications

Gomez, SR., Xing, DK., Corbel, MJ., Coote, J., Parton, R., Yuen, CT. (2006) Development of a carbohydrate binding assay for the B-oligomer of pertussis toxin and toxoid. *Analytical Biochemistry*. **356**: 244-253

Gomez, SR., Yuen, CT., Asokanathan, C., Douglas-Bardsley, A., Corbel, MJ., Coote, JG., Parton, R., Xing, DK. ADP-ribosylation activity in pertussis vaccines and its relationship to the in vivo histamine-sensitisation test. *Vaccine*. In press

Gomez, SR., Yuen, CT., Asokanathan, C., Douglas-Bardsley, A., Corbel, MJ., Coote, JG., Parton, R., Xing, DK Comparison of *in vivo* and *in vitro* activities of pertussis toxin and toxoid 11th European workshop on bacterial toxins, Prague (28th June 2003- 02-July 2003)

Gomez, SR., Xing, D., Parton, R., Coote, J., Corbel, MJ., Yuen, CT. A novel ELISA for determining the binding activities of pertussis toxin. 12th European workshop on bacterial toxins, Kent, UK (25th June 2005- 29th-June 2005)

Wheeler, J., Gomez, SR., Jones, J., Yuen, C-T., Corbel, M and Xing, D. Proteomic analysis on effect of pertussis toxin on CHO cells using two-dimensional gel electrophoresis. HPA annual conference, Warwick, UK (12th Sept-14th Sept 2005)

Chapter 1

Introduction

1.1 The pathogen, the disease and treatment

The genus *Bordetella* currently contains nine species mostly pathogenic for different host organisms and typically associated with upper respiratory tract infections, with the exceptions of *B. trematum*, *B. petrii* and *B. ansorpii* (Preston, 2005; Ko *et al.*, 2005). *B. pertussis* is exclusively a human pathogen and was first described as the causative agent of pertussis (whooping cough) in 1906 by Bordet and Gengou. The organism is a small fastidious Gram-negative coccobacillus arranged in singly or in pairs and shows affinity for mucosal layers of the human respiratory tract (Figure 1.1) (Parton, 1998). *B. pertussis* shares many features with its related species in that it expresses a common set of adhesins and toxins required for colonisation and virulence. However, it is unique in expressing pertussis toxin (PT), a molecule that has been reported to be an important virulence factor and immunogen (Sato *et al.*, 1984; Weiss *et al.*, 1984; Preston *et al.*, 2004).

Pertussis is a highly communicable disease lasting for 6 to 12 weeks or longer. It is divided into three distinctive clinical stages: catarrhal, paroxysmal, and convalescent. Initially, pertussis starts with catarrhal symptoms (coryza, conjunctivitis, occasional cough, and minimal or no fever) that are indistinguishable from those of other minor upper respiratory infections. Over a 7 to 14 day period the cough becomes paroxysmal. Paroxysms are characterised by repeated coughing fits with 5 to 10 or more forceful coughs during a single expiration often followed by a single sudden massive inspiration. This inspiration produces the characteristic whooping sound and is the hallmark of the disease. Cyanosis and post-tussive vomiting is common. Leucocytosis with absolute lymphocytosis is also characteristic in a typical case. The paroxysmal phase is

the most dangerous with the greatest risk of complications including pneumonia, seizures and encephalopathy (Wortis *et al.*, 1996; WHO position paper, 2005). The paroxysmal stage lasts for 2 to 8 weeks or more and, if individuals survive the paroxysmal stage, it is followed by the gradual transition to the convalescent stage. During this stage there is a gradual decline in all symptoms. The paroxysms, however, can occur sporadically for as long as 6 months after the infection (Cherry, 1999; Mattoo and Cherry, 2005). Clinical manifestations vary by age and both immunisation and previous infection moderate the severity of the clinical symptoms (Greenberg, 2005; Yarri *et al.*, 2006).

Diagnosis of pertussis can be made by direct detection of the bacteria by culture, polymerase chain reaction or by serological methods (WHO position paper, 2005). Pertussis treatment and post-exposure prophylaxis is usually by a course of erythromycin, clarithromycin or azithromycin. Trimethoprim-sulphamethoxazole can be used as an alternative if the patient cannot tolerate macrolides or is infected with a macrolide-resistant strain of *B. pertussis* (Tiwari *et al.*, 2005). Use of antibiotic is most effective if commencement is during the catarrhal stage although diagnosis at this early stage is difficult since initial symptoms are non-specific. In the paroxysmal phase of the disease, the use of antibiotics rarely affects the course of the disease but can eradicate any secondary pulmonary infection and *B. pertussis* from the nasopharynx, making the patient non-infectious to others. (WHO Model Prescribing Information, 2001; The US Center for Disease Control and Prevention (CDC), 2005a). The most effective method to control the global burden of pertussis has been through immunisation with efficacious preparations of whole-cell and acellular pertussis vaccines: 'prevention is better than treatment' (Erasmus (1466-1536) (sections 1.2 and 1.5).

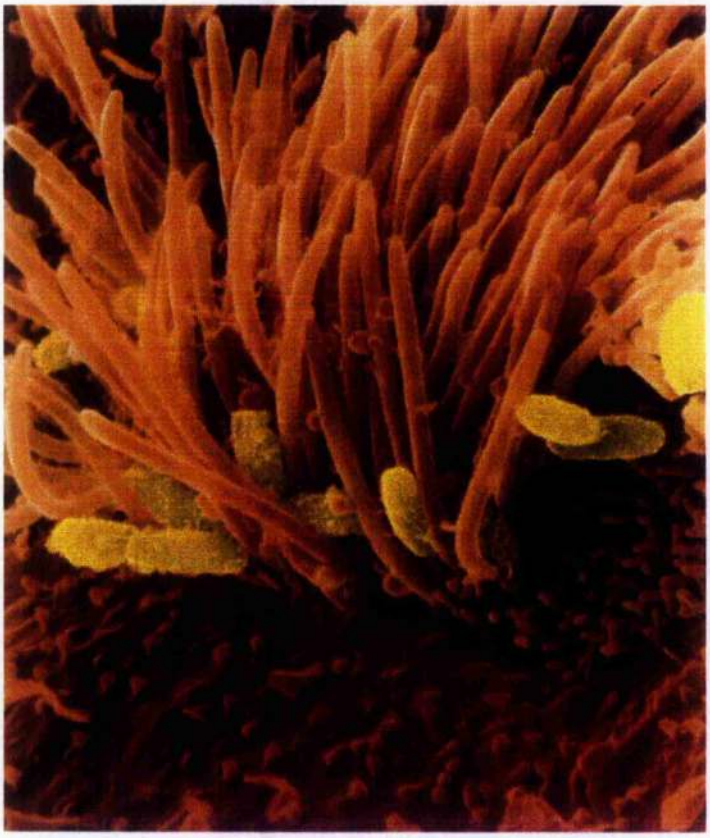


Figure 1.1. Attachment of *B. pertussis* to the ciliated epithelium of the respiratory tract Taken from NIBSC photographic library. Green, *B. pertussis*; red, epithelial cells; and orange, cilia.

1.2 Epidemiology and prevention of pertussis

In the pre-vaccine era, pertussis was among the most common childhood diseases causing high mortality rates in children less than 5 years of age (Cherry, 1999). The introduction and widespread use of pertussis vaccines markedly decreased the incidence to low levels in countries with good vaccine coverage. The effectiveness of pertussis vaccines was further corroborated with observed epidemics during the periods when there was a decline or withdrawal of vaccines due to concerns regarding safety and efficacy of pertussis vaccines (Cherry, 1988). Despite the availability of vaccines, pertussis remains a major public health problem. Recent estimates from WHO suggest that in 2003, about 17.3 million cases of pertussis occurred worldwide and 279 000 fatalities, with the highest incidence rates (90%) occurring in the developing countries (WHO position paper, 2005). In addition, resurgence of pertussis has been observed in many countries with high vaccine coverage and has indicated an epidemiological shift in the age distribution to adolescents and adults. The US National surveillance data from CDC found that the annual incidence of pertussis among persons aged 10-19 increased from 5.5% to 10.9% from 2001 to 2003. Similar increases were found in Canada, Australia and some European countries (WHO position paper, 2005; CDC, 2005b, 2006; Celentano *et al.*, 2005; Greenberg, 2005). Possible reasons include increased recognition of the disease, quality of vaccines, waning immunity following previous infection or immunisation and possibly the antigenic variation between circulating strains and vaccine strains (Bentsi-Enchill *et al.*, 1997; Mooi *et al.*, 1999; Melker *et al.*, 2000; Mooi *et al.*, 2001; Gzyl *et al.*, 2004; Godfroid *et al.*, 2005). Re-infections with pertussis in adults are a significant health burden since they are likely to transmit the disease to infants who are too young for immunisation or not fully protected. For this reason, several countries have implemented the US Food and Drug Adminstration

(FDA) approved booster immunisation with acellular pertussis vaccines for adolescents and adults (Greenberg, 2005). The worldwide introduction of booster doses in adolescents and adults is expected in the near future. Since immunity after vaccination is short-lived, an interval of 10 years between booster doses has been recommended which would decrease *B. pertussis* disease rates in these groups and hence transmission (Forsyth *et al.*, 2005).

1.3 Pathogenicity of *B. pertussis*

There are several features that make *B. pertussis* and other bacterial infectious agents pathogenic to humans. Disease is preceded by the attachment of the organism to the specific target tissue of the host (colonisation) followed by local tissue damage, secretion of products required for causing systemic disease and inhibition or evasion of host immune defences. *B. pertussis,* unlike the agents of some other diseases such as those causing diphtheria and tetanus, in which the clinical manifestations are mediated by a single toxin, is sophisticated in that it produces a vast number of virulence factors classically divided into adhesins and toxins that are likely to interact in a complex manner to establish infection (Figure 1.2).

1.3.1 Regulation of virulence factors

Expression of most of the virulence factors of *B. pertussis* is regulated in response to certain environmental stimuli, by the BvgA/S phosphorelay two-component signal tranduction system. This comprises the environmental sensor transmembrane protein BvgS and the DNA-binding response-regulator protein BvgA (reviewed by Locht, 1999; Matoo and Cherry, 2005). BvgS contains a periplasmic domain, a linker region, a

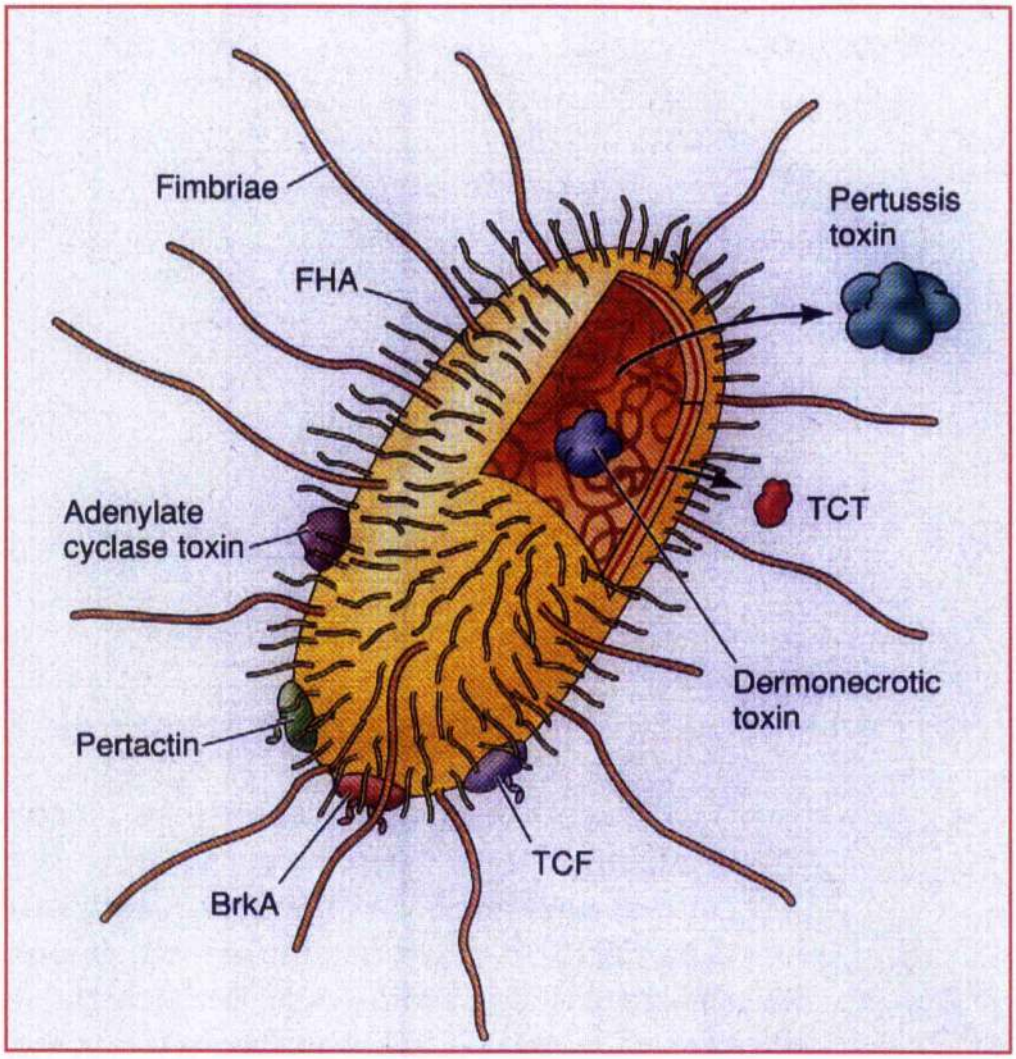


Figure 1.2. Typical virulence factors associated with the pathogenesis of pertussis. Toxins include secreted pertussis toxin (PT), tracheal cytotoxin (TCT), surface associated (mainly) adenylate cyclase toxin and cytoplasmic dermonecrotic toxin. Attachment factors include filamentous haemagglutinin (FHA), pertactin, *Bordetella* resistance to killing factor A protein (BrkA), tracheal colonisation factor (TcfA) and fimbriae. Taken from Weiss (1997).

transmitter, a receiver, and a histidine phosphotransfer domain (HPD). At 37^0C and in the absence of $MgSO_4$ and nicotinic acid, BvgS autophosphorylates and relays phosphorylation from the periplasmic domain to the HPD of BvgS. The HPD can transfer the phosphate back to the BvgS or phosphorylate BvgA. The phosphorylation of BvgA promotes the transcription of *vag* genes (virulence-activated genes) which encode most of the virulence factors of *B. pertussis*. At the same time BvgA activates an intermediate regulatory gene BvgR which represses the expression of *vrg* genes (virulence-repressed genes).

1.3.2 Adhesins

1.3.2.1 Filamentous haemagglutinin (FHA)

FHA is encoded by the *fhaB* gene and is synthesised as a large precursor protein of 367 kDa and undergoes N- and C- terminal modifications to form the mature 220 kDa FHA protein. It is exported across the cytoplasmic membrane via the *B. pertussis* Sec machinery (Lambert-Buisine *et al.*, 1998) and its translocation to the outer membrane requires accessory protein *fhaC* (Jacob-Dubuison *et al.*, 1999, 2001; Guedin *et al.*, 2000). After expression on the cell surface, mature FHA protein is released following proteolytic cleavage by subtilisin-like autotransporter/protease, SphB1 (Coutte *et al.*, 2001). Although FHA is secreted, it is also found to be associated with the cell surface.

FHA has multiple binding specificities that may explain its differential roles in pathogenicity. It has two distinctive domains, which mediate attachment to ciliated and non-ciliated respiratory cells via lactosylceramides and sulphated glycosaminoglycans respectively (Menozzi *et al.*, 1991; Prasad *et al.*, 1993). In addition, it contains an Arg-

Gly-Asp (RGD) motif that has been shown to stimulate adherence to monocytes/macrophages by interaction with a complex composed of leukocyte response integrin and integrin-associated protein, resulting in the up-regulation of CR3 binding activity (Relman *et al.*, 1990; Ishibashi *et al.*, 1994, 2002; and Mobberley-Schuman and Weiss, 2005). CR3 is also one of the receptors for FHA. Although FHA mediates the phagocytosis and potential self-destruction of *B. pertussis* by binding to CR3 receptor, its cooperation with adenylate cyclase toxin (discussed later) prevents the oxidative burst (Mobberley-Schuman and Weiss, 2005). This may explain the persistence of pertussis in patients and intracellular survival of *B. pertussis*. In summary, FHA primarily acts as an adhesin and cooperates with other *B. pertussis* components to mediate both colonisation and immune evasion. During infection in both human and animal models, FHA produces a strong IgA and IgG antibody response (Thomas *et al.*, 1989). Vaccination with purified FHA protects mice against *B. pertussis* infection and moreover, clinical studies have shown that two component (FHA and PTd) acellular pertussis vaccines are more effective than monocomponent PTd vaccines (Cherry, 1997). For these reasons, FHA is considered to be an important component in acellular-based pertussis vaccines (Sato *et al.*, 1984; Sato and Sato, 1985). Nevertheless it is noteworthy that a clear correlation between serum antibody responses to FHA and protection in children has not been found (Cherry *et al.*, 1998).

1.3.2.2 Fimbriae (Fim)

B. pertussis produces serologically distinct fimbriae of two serotypes, designated serotype 2 and serotype 3, and these are composed of major subunits Fim2 (22.5kDa) and Fim3 (22kDa) respectively. In addition to the major subunits, both fimbriae also contain a minor 40kDa subunit, FimD, which is located at the fimbrial tip (Zhang *et al.*,

1985; Irons *et al.*, 1985; Willems *et al.*, 1993; Geuijen *et al.*, 1996). Expression of the *fim2* and *fim3* genes is regulated by the bvgA/S system and, and at an individual level, by a phenomenon called phase variation. The latter occurs due to deletions or insertions at the promoter region which results in strains producing both types of fimbriae, one type or none at all (Willems *et al.*, 1990). This feature is clinically significant since phase variation has been attributed to the observed adaptation of circulating *B. pertussis* strains, presumably due to immunological pressures (Preston, 1980; Willems *et al.*, 1990). For example, numerous workers have demonstrated that vaccines lacking in one or other serotype components can result in pertussis cases in individuals previously immunised, caused by the missing serotype (Preston *et al.*, 1985; Tiru *et al.*, 1997).

Like FHA, both major and minor fimbrial subunits have been shown to bind to sulphated glycoconjugates, which are ubiquitous in the respiratory tract, and have been implicated in the colonisation state of the disease (Geuijen *et al.*, 1996, 1997; Rodriguez *et al.*, 2006). FimD also binds to the integrin VLA-5, which activates CR3, the receptor for FHA. It is believed that, *in vivo*, fimbriae act in synergy with FHA (Hazenbos *et al.*, 1995). In line with this, the gene encoding fimD is part of a gene cluster involved in fimbrial and FHA biosynthesis (Willems *et al.*, 1993,1994). Fimbriae have been shown to be protective immunogens in mice, and are also included in some acellular pertussis vaccine formulations. These preparations have also been shown to have higher efficacy than vaccines containing only PTd, FHA and PRN (Olin *et al.*, 1997).

1.3.2.3 Pertactin (PRN), tracheal colonisation factor A (TcfA) and *Bordetella* resistance to killing factor A protein (BrkA)

These proteins belong to the autotransporter family of proteins in Gram-negative bacteria. They are structurally similar and are all synthesised as pre-proteins. They have an N-terminal signal sequence to direct translocation across the inner membrane, a passenger domain that is responsible for effector functions of the protein and a C-terminal domain that mediates secretion across the outer membrane (Henderson *et al.*, 2001). Mature pertactin is a 69 kDa protein and is localised predominantly in the outer membrane. Like FHA, pertactin contains an RGD tripeptide motif as well as several proline-rich regions and leucine-rich repeat motifs that are characterisitic of molecules that form protein-protein interactions involved in eukaryotic cell binding (Emsley *et al.*, 1994). Pertactin in combination with other *B. pertussis* adhesins probably contributes to the pathogenesis of pertussis via its involvement in bacterial adhesion. BrkA is expressed as a 103kDa pre-protein that is processed to yield a 73 kDa passenger domain and 30 kDa C terminal (Oliver *et al.*, 2003a,b). It is a cell surface-associated protein and, similar to FHA and PRN, has two RGD sequences and has been shown to mediate adhesion to or invasion of a variety of cells (Fernandez *et al.*, 1994). In addition it enables the bacterium to resist the bactericidal activity of normal human serum by the antibody-dependent complement pathway and of some antimicrobial peptides (Fernandez *et al.*, 1994; Fernandez and Weiss, 1996). Tracheal colonisation factor A (TcfA) is a secreted 60 kDa protein. Its sequence also has proline-rich regions and like FHA, pertactin and BrkA, it also has an RGD sequence which appears to be involved in the colonisation of the trachea (Finn and Steven, 1995). All of these three proteins appear to contribute to the colonisation of *B. pertussis* but only PRN is currently present in commercially-available acellular pertussis vaccines and has been shown to be an

important protective antigen in animal models and following immunisation in humans. In fact, antibody levels against PRN correlated with protection in humans against pertussis (Olin *et al.*, 2001). In addition, vaccines containing PT, FHA and PRN are superior to vaccines containing only PT and FHA (Gustafsson *et al.*, 1996; Cherry, 1997).

1.3.3 Toxins

1.3.3.1 Adenylate cyclase toxin (ACT)

ACT is a large, bifunctional protein of 1706 amino acid residues encoded by the *cyaA* gene. Its calmodulin-dependent-adenylate cyclase (cytotoxic) activity resides in the N-terminal 400 amino acids whereas its haemolytic (pore forming) activity is due to the 1300 amino acid C-terminal region (Glaser *et al.*, 1988), which is also essential for the delivery of the catalytic domain into the cytoplasm of eukaryotic cells (Coote, 1996). Once inside the host cell, the catalytic domain is activated by endogenous calmodulin and catalyses the uncontrolled production of cAMP from ATP and subsequent intoxication of target cell (Wolff *et al.*, 1980). The physiological consequences include the inhibition of neutrophil functions, including chemotaxis, oxidative burst and phagocytosis, and it also induces apoptosis in cultured murine macrophages (Confer and Eaton, 1982; Friedman *et al.*, 1987; Khelef *et al.*, 1993; Njamkepo, 2000). The intoxication of phagocytic cells by ACT is mediated by its specific binding to CD11b/CD18 integrin (Guermonprez *et al.*, 2001). However, ACT at high concentrations has also been shown to intoxicate cells that do not bear these receptors, by penetrating the lipid bilayer (Martin *et al.*, 2004). Since ACT mainly affects immune cells, it is believed to contribute to the pathogenicity of *B. pertussis* by evasion of host

immune defences and, in conjunction with other virulence factors, in providing an intracellular niche for the survival and persistence of *B. pertussis* infection (Friedman *et al.*, 1992; Hellwig *et al.*, 1999). Although ACT has been shown to induce a high specific antibody level after primary infection, to display adjuvant activities to co-administered antigens when using both native ACT and detoxified preparations, and to be a protective antigen in the mouse models, it has yet to be included in any acellular vaccine (Guiso *et al.*, 1991; Cherry *et al.*, 2004; Macdonald-Fyall *et al.*, 2004).

1.3.3.2 Dermonecrotic toxin (DNT)

As the name suggests, DNT causes skin lesions in a variety of animals and is lethal following intravenous injection in mice at high doses (Wardlaw and Parton, 1983; Livey and Wardlaw, 1984; Parton, 1985). DNT is a single polypeptide of 160 kDa (calculated mass) consisting of an N-terminal receptor-binding domain and a C-terminal enzymatic domain (Schimdt *et al.*, 1999; Kashimoto *et al.*, 1999; Matsuzawa *et al.*, 2002). The full expression of enzymatic activity *in vitro* requires the prior binding of DNT to uncharacterised receptors followed by internalisation by dynamin-dependent endocytosis into endosomes where it undergoes proteolytic processing before the translocation of the enzymatic domain to the cytosol (Matsuzawa *et al.*, 2004). DNT has transglutaminase activity and deamidates and polyaminates Gln63 of Rho GTPases (Gln61 of Rac and Cdc42) that impair GTP hydrolysis, rendering the constitutive expression of Rho GTPases (Horiguchi *et al.*, 1995, 1997; Schmidt *et al.*, 1999; Masuda *et al.*, 2002). Some of the effects associated with activation of Rho GTPases by DNT include morphological changes associated with actin fibre assembly and formation of focal adhesions, stimulation of DNA and protein synthesis, inhibition of osteoblastic differentiation and adjuvant activity (Horiguchi *et al.*, 1991; Horiguchi *et al.*, 1993;

Horiguchi *et al.*, 1995; Horiguchi *et al.*, 1997; Munro *et al.*, 2005). How these factors contribute to the pathogenesis of whooping cough is largely unknown and has not been studied well since mutants lacking the DNT appear to be as virulent as the wild type in a mouse model of the disease (Weiss *et al.*, 1989).

1.3.3.3 Lipopolysaccharide (LPS)

B. pertussis is unusual in that it produces two distinct types of LPS molecules: Type A consists of lipid A plus a branched core oligosaccharide and a trisaccharide consisting of α-*N*-acetylglucosamine, β-2-acetamido-3-acetamido-2,3-dideoxy-mannuronic acid and β-1-2 acetamido-4-methylamino-fucose; Type B contains lipid A and the oligosaccharide core structure (Peppler *et al.*, 1984; Caroff *et al.*, 2000). The expression of the trisaccharide is controlled by the 12-gene *wlb* operon and is regulated by the BvgA/S system (Allen *et al.*, 1996, 1998). In general, like endotoxin from other Gram-negative bacteria, *B. pertussis* LPS is toxic, pyrogenic, mitogenic in spleen cell cultures and can activate macrophages and induce tumor necrosis factor production (Watanabe *et al.*, 1990). Some of these factors may be the cause of mild fever seen in some pertussis cases. Additional roles that have been elucidated by mutational analysis in the *wlb* locus and include colonisation and persistence within the mouse respiratory tract and evasion of host immune defences (Harvill *et al.*, 2000; Schaeffer *et al.*, 2004).

1.3.3.4 Tracheal cytotoxin (TCT)

TCT is a disaccharide-tetrapeptide of 921 Da fragment released from the *Bordetella* peptidoglycan during growth or bacterial lysis. (Rosenthal *et al.*, 1987; Cookson *et al.*, 1989). It is always expressed and independent of BvgAS control. TCT is the only toxin

that has been shown to reproduce the cytopathology attributed to *B. pertussis* infection and explain the paroxysmal coughing (Wilson *et al.*, 1991). It causes ciliostasis and extrusion of ciliated cells in hamster tracheal ring cultures, which will impair normal lung clearance of debris (Goldman *et al.*, 1985). These effects are thought to be associated with the TCT-dependent increased production of intracellular interleukin-1α (IL-1α) and subsequent production of nitric oxide (Heiss *et al.*, 1993; Flak and Goldman., 1999; Flak *et al.*, 2000).

1.3.3.5 Pertussis toxin (PT)

This toxin will be discussed in the following section in more detail as its study forms a major part of this thesis.

1.4 PT

1.4.1 Structure and biogenesis

PT is an A-B toxin consisting of six polypeptides, S1 (26,220 Da), S2 (21,920), S3 (21,860), S4 (12,060) and S5 (10,940) in a 1:1:1:2:1 ratio. The A-subunit comprises the S1 polypeptide and is responsible for the enzymatic activity whereas the B-subunit, mediating binding and possibly intracellular translocation, consists of polypeptides S2 to S5 (Tamura *et al.*, 1982). Each polypeptide is encoded separately and contains a classical N-terminal signal sequence (a stretch of hydrophobic amino acids), indicating transport into the periplasmic domain probably via a general export pathway (Nicosia *et al.*, 1986; Locht *et al.*, 1986; Weiss *et al.*, 1993; Stathopoulos *et al.*, 2000). Once the polypeptides are exported across the inner membrane of *B. pertussis* to the periplasm,

the S1 localises to the outer membrane and serves as a site for the assembly with the B-oligomer (Farizo *et al.*, 2002). The correct assembly of polypeptides to form the holotoxin is critical for the secretion of toxin since A- and B-subunits alone were not secreted (Farizo *et al.*, 2000; Craig-Mylius *et al.*, 2000; Stenson and Weiss, 2002). Crystal structure analysis of the assembled PT revealed that the toxin forms in the shape of a pyramid with the enzymatic S1 subunit sitting on top of its ring-like structure formed by the cell-binding B-oligomer, S2-S5 (Figure 1.3). Each of the five subunits consists of a common folding motif of approximately 100 residues that consists of the six anti-parallel β-strands forming a closed β-barrel, capped by an α-helix. The B-oligomer is arranged as two dimers, D1 (S2-S4) and D2 (S3-S4) joined together by S5 subunit. The ring-like structure is formed by the association of B-subunits predominantly through anti-parallel β-sheet interactions to form an asymmetrical pentamer surrounding a central pore formed by five helices. The carboxyl terminus of S1 penetrates into the central pore (Tamura *et al.*, 1982; Stein *et al.*, 1994a). Secretion of PT from *B. pertussis* is a complex process requiring the products of nine *ptl* (pertussis toxin liberation gene) genes (*ptlA-I*) that are located downstream of the structural genes (Kotob *et al.*, 1995; Farizo *et al.*, 1996). The *ptl* genes share considerable homology with the virB genes of *Agrobacterium tumefaciens*, which encode the type IV family of secretion systems that are involved in exporting virulence factors across the membranes of pathogenic bacteria. It is thought that PT may be secreted from *B. pertussis* in a similar manner (Weiss *et al.*, 1993; Burns, 2003).

1.4.2 Molecular mechanisms of toxin action

PT is considered a major virulence factor produced by *B. pertussis* and is well known to cause a wide range of physiological effects including histamine sensitisation,

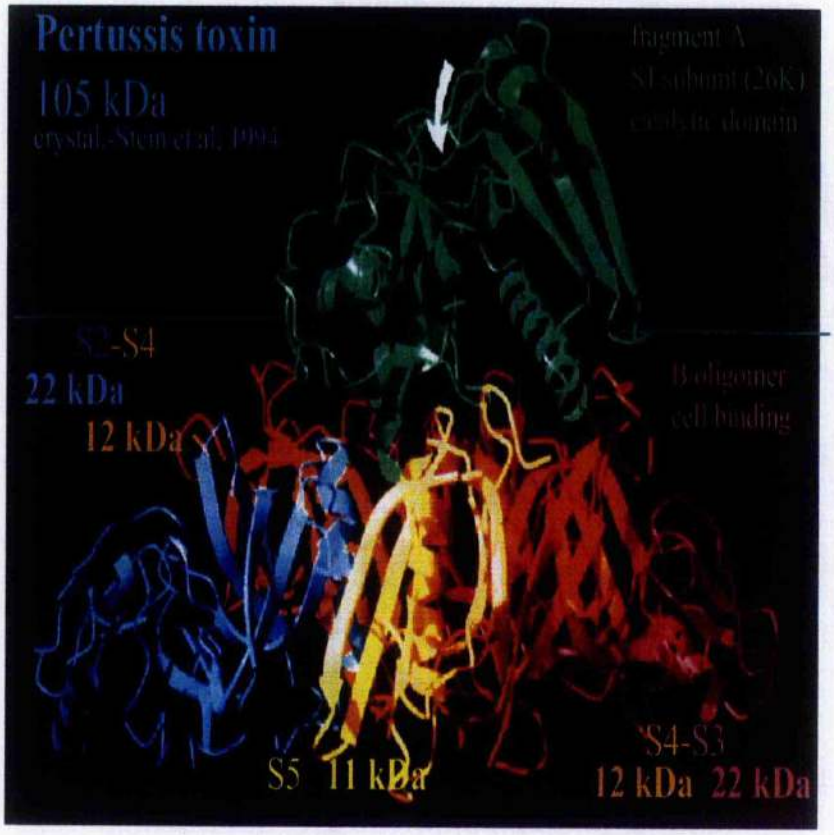


Figure 1.3. Schematic picture of PT. Taken from Stein *et al.*, 1994a.

hyperinsulinaemia, lymphocytosis promoting activity, adjuvant effects, clustering of Chinese Hamster Ovary (CHO) cells, mitogenesis, haemagglutination activities, stimulation of lipolysis in rat adipocytes and increased permeability in the blood brain barrier (Munoz, 1985; Bruckener *et al.*, 2003). The majority of the biological activities are attributed to the enzymatic activity of the S1 subunit but some are dependent on the B-subunit activities alone. Following secretion by *B. pertussis,* PT needs to enter the membrane of the target host cell before carrying out its various activities. This is believed to involve three stages. 1) Binding of the B-oligomer to host cells, 2) internalisation and translocation of the S1 subunit to the cytosol, and 3) ADP-ribosylation of G proteins catalysed by the S1 subunit.

1.4.2.1 Binding

PT is clearly a complex molecule binding to a vast number of cell types derived from human and animal sources including bronchial epithelial cells, CHO cells, pancreas derived cells, endothelial derived cells, kidney derived cells, erythrocytes from chicken and goose, platelets, adipocytes, T and B lymphocytes and macrophages (Tamura *et al.*, 1983; Sekura and Zhang, 1985; Kolb *et al.*, 1990; Rogers *et al.*, 1990; Saukkonen *et al.*, 1992; Sindt *et al.*, 1994; Armstrong *et al.*, 1994; el Baya *et al.*, 1999). Although the majority of the cell types studied have been shown to express PT binding proteins, a unique receptor for PT remains elusive. However, several lines of evidence suggest that sialylated and non-sialylated carbohydrates are crucial components of receptors recognised by the toxin (Sekura and Zhang, 1985; Capiau *et al.*, 1986; Brennan *et al.*, 1988; Saukkonen *et al.*, 1992; van't Wout *et al.*, 1992; Spangler *et al.*, 1993; Menozzi *et al.*, 2002). In addition, *N*-linked oligosaccharides and terminal sialic acid residues appear to be important for toxin recognition (Armstrong *et al.*, 1988; Witvliet *et al.*,

1989; Hausman and Burns, 1992; el Baya *et al.*, 1999). For example, the ability of PT to ADP-ribosylate G proteins in intact CHO cells and its subsequent cytotoxic effect (clustering) was significantly altered in a CHO cell line specifically lacking terminal sialic acid (NeuAc) or carbohydrate sequences NeuAc→Galβ4 or NeuAc→Galβ4GlcNAc on complex type glycoproteins (Brennan *et al.*, 1988; Witvliet *et al.*, 1989). Since the mutant CHO cells only differ from wild type cells in the structure of their *N*-linked oligosaccharide chain, the receptor for PT is likely to be a glycoprotein. Consistent with the lectin-like properties of PT, crystal structural analysis revealed regions in the B-oligomer that show structural homology to the family of calcium-dependent eukaryotic lectins and wheat germ agglutinin, both of which are carbohydrate binding proteins (Stein *et al.*, 1994).

PT binds to some serum sialo-glycoproteins like haptoglobin and fetuin (Tamura *et al.*, 1983; Sekura and Zhang, 1985) and these have been used as ligands in affinity chromatographic columns for purification of the toxin (Capiau *et al.*, 1986; Sekura *et al.*, 1983). Apart from glycoproteins, PT also binds to glycolipids such as lactosylceramide and gangliosides (Saukkonen *et al.*, 1992; Hausman *et al.*, 1993). However, quartz crystal microbalance measurements investigating direct binding of PT with gangliosides did not support strong binding to glycolipids (Janshoff *et al.*, 1997). The binding of PT to glycoproteins and glycolipids is mediated by the S2 and S3 subunits (Witvliet *et al.*, 1989; Schmidt *et al.*, 1989., 1991; Saukkonen *et al.*, 1992). Experimental evidence suggests there are at least two different classes of carbohydrate structures recognised by the S2 and S3 subunits: α-2,6-linked sialic acids and *N*-acetyllactosamine residues (Tuomanen *et al.*, 1988; Brennan *et al.*, 1988; Armstong *et al.*, 1988; Witvliet *et al.*, 1989; Heerze and Armstrong., 1990; Hausman and Burns., 1993). However, the extent of binding to specific oligosaccharide structure(s) mediated

by S2 and S3 is unclear (Lang *et al.*, 1989; Witvliet *et al.*, 1989; Schmidt *et al.*, 1989; Loosmore *et al.*, 1993; Lobet *et al.*, 1993; Raze *et al.*, 1998).

The binding of PT to fetuin, a glycoprotein consisting of *O*- and *N*- linked glycans has been used as a model system to characterise the binding specificities of PT. Using sequential degradation and reglycosylation experiments it was found that PT binds better to oligosaccharides terminated with α2,6-linked NeuAc than *N*-acetamido sugars and binds least with galactose residues (Sekura and Zhang, 1985; Armstrong *et al.*, 1988). A study using mutant CHO cells with specific *N*-glycosylation defects, showed that optimum binding of PT required a complete sialylactosamine chain (Witvliet *et al.*, 1989). So far, the relative binding of PT to complex oligosaccharides with defined structures using a direct binding assay has not been investigated.

1.4.2.2 Entry

The molecular mechanisms involved in the entry and translocation of S1 subunits to the cytosol of host target cells and relative roles of both A- and B-subunits in this process is so far unclear. However, several studies indicate that internalisation is by endocytic uptake involving early/late endosomes and Golgi apparatus (Xu and Barbieri, 1995; Xu and Barbieri, 1996; el Baya *et al*, 1997) and possibly the endoplasmic reticulum (ER) (Hazes *et al.*, 1996; Hazes and Read, 1997; Carbonetti *et al.*, 1999; Veithen *et al.*, 2000; Castro *et al.*, 2001). Consistent with the involvement of the ER, ATP, which is required for the *in vitro* activation of PT, is only present in the ER and not other cytosolic compartments involved in the endocytic pathway. ATP binds to the B-oligomer and permits the dissociation of S1 from the B-oligomer (Lim *et al.*, 1984; Burns and Manclark, 1989; Hausman *et al.*, 1990; Hausman and Burns, 1992; Hazes *et al.*, 1996). It has been proposed that dissociation of AB-subunits allows the S1 subunit to disguise

itself as misfolded protein (i.e by exposure of hydrophobic regions and reduction of disulphide bond) and to use the endoplasmic reticulum (ER)-associated degradation pathway to access the cytoplasm where it escapes ubiquitination due to lack of lysine residues (Hazes and Read, 1997). Previous studies have demonstrated that efficient binding of PT and PT S1 subunit to model phospholipid vesicles required the presence of ATP and a thiol reductant respectively (Burns and Manclark, 1989; Krueger and Barbieri, 1993; Hausman and Burns, 1992). Crystal structure analysis studies also indicated that the proposed activation (reduction) regions on the PT molecule would result in a conformational change that would result in the exposure of hydrophobic regions (Stein *et al.*, 1994a).

Alternatively, PT may directly traverse the membrane (Spangler *et al.*, 1993; Kaslow and Burns, 1992). Crystal structure analysis of PT complexed with oligosaccharide structures derived from transferrin indicated that sialic acid binding sites are located near the S1. Based on this observation it was suggested that the orientation of the holotoxin after binding is such that it brings the S1 close to the membrane (Stein *et al.*, 1994b). Considering the ability of PT to affect many different cells types and the fact that binding is mediated by either S2 or S3 or both S2 and S3 depending on target cell, internalisation and translocation process could also be cell-type specific.

1.4.2.3 ADP-ribosyl transferase activity

Once the S1 subunit has reached the cytoplasmic side of the host cell it catalyses the transfer of the ADP-ribose moiety from NAD to the cysteine residue located 4 amino acids from the carboxyl terminus of the α-subunit of guanine nucleotide-binding proteins (G-proteins) which include G_i, G_0, and G_t (Ui, 1990). The catalytic activity

resides in the amino terminal region (1-180) of the S1 subunit as determined by mutagenesis studies (Pizza *et al.*, 1988; Locht *et al.*, 1989; Loosmore *et al.*, 1990). G-proteins act as molecular switches for many biological processes. In their inactive state, G-proteins are a trimeric form containing GDP bound to the α–subunit and attached either to the inner surface of the plasma membrane or to the inner surface (cytosolic side) of the transmembrane receptors (known as G-protein coupled receptors) (Figure 1.4). Upon external stimuli, the receptor undergoes a conformational change causing the α–subunit of G-protein to mediate exchange of its GDP to GTP. This then triggers the dissociation of the trimeric form to Gα and Gβγ. The Gα is now active and interacts with the appropriate effector protein. For example, the Giα protein interacts with adenyl cyclase and causes inhibition of cAMP synthesis (Ui, 1990). The Gα protein has intrinsic GTP hydrolase activity which exchanges the GTP for GDP and in doing so results in the dissociation from the effector protein and reassociation with the Gβγ dimer, hence returning to the inactive state. PT catalysed modification of G-proteins prevents the exchange of GDP for GTP thus, if the G_i is targeted, the inhibitory pathway is blocked (Krueger and Barbieri, 1995). The biological consequences of ADP-ribosylation by PT includes histamine sensitisation, leucocytosis/lymphocytosis, hyperinsulinaemia with subsequent hypoglycaemia and CHO cell clustering (Sekura *et al.*, 1985), which will be discussed in the following section.

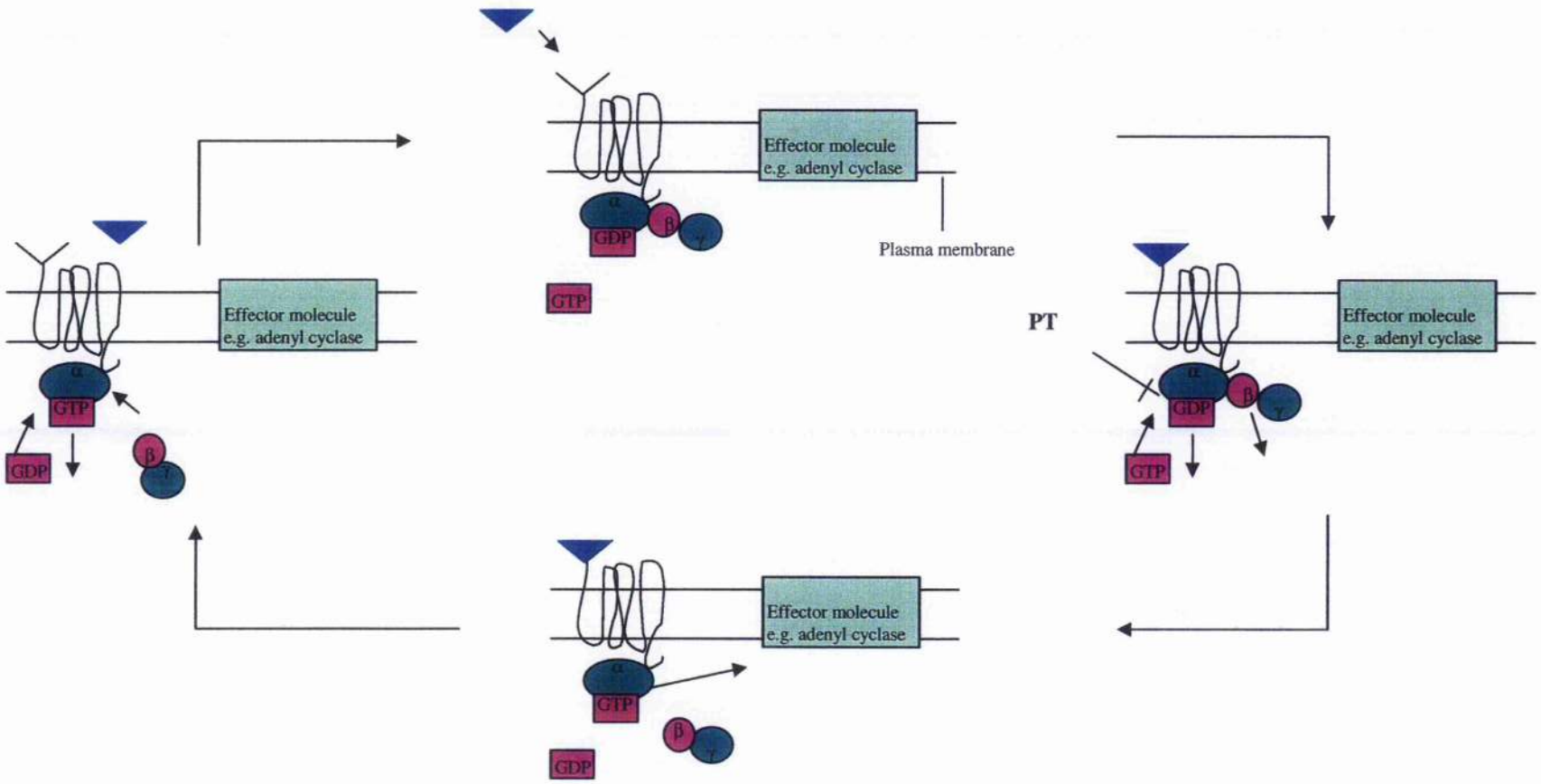


Figure 1.4. Schematic representation of G-protein-coupled receptor signalling and the mechanism of PT action. In its inactive state Gα has GDP in its binding site. The occupation of the receptor with an agonist allows the GDP to be displaced by GTP. GTP activates Gα causing it to dissociate from GβGγ (which remain linked as a dimer). Activated Gα in turn activates the effector molecule. PT ADP-ribosylates the α-subunit preventing the exchange of GDP for GTP, therefore the effector pathway is blocked. Adapted from Krueger and Barbieri (1995)

1.4.3 Biological activities of PT

PT was originally given several names based on its diverse biological activities. It was only after extensive purification and characterisation that it was realised that the vast array of biological activities was attributable to a single protein (Munoz, 1985). Although the precise mechanisms of many of the biological activities are still obscure, with the progression of molecular tools, it is now known that some of the effects are a direct consequence of catalytic activity and others are a result of the B-subunit activity. Some of the most relevant biological activities of PT will be discussed in the following sections.

1.4.3.1 Lymphocytosis promoting activity

The pronounced leucocytosis, predominantly as lymphocytosis, seen in pertussis cases is one of the hallmarks of the disease and one that is exclusively associated with PT. It is characterised by the increase in small lymphocytes, that include both T-cells and B-cells, also polymorphonuclear leukocytes, a marked decrease in the weight of the thymus and lymph nodes and an increase in the spleen weight (Morse and Morse, 1976; Munoz, 1985). The observed increase in lymphocytes is not due to proliferation but is due to the prevention of the recirculation of these cells through the lymphoid tissue (Morse and Riester, 1967; Spangrude *et al.*, 1984). Further to this, the immunophenotype of peripheral blood lymphocytes derived from pertussis cases relative to uninfected subjects showed a marked decrease in L-selectin but the mechanisms remain elusive. L-selectin is an adhesion molecule essential for lymphocyte homing (Hudnall and Molina, 2000; Hodge *et al.*, 2003). Lymphocytosis is primarily dependent on the expression of the catalytic activity of PT. However, there are studies showing that the B-oligomer also contributes since recombinant PT with

alterations in the B-oligomer demonstrated reduced lymphocytosis (Loosmore *et al.*, 1993; Raze *et al.*, 1998). The number of circulating lymphocytes in the whole blood of immunised mice can be used as a check for determining the level of active PT in pertussis-based vaccines (Section 1.6.2.1)

1.4.3.2 Hyperinsulinaemia with subsequent hypoglycaemia

One of the original names of PT was 'islet activating protein' as it had a unique action of enhancing insulin secretory responses of experimental animals to nutritional and hormonal stimuli (Yajima *et al.*, 1978). Early observations showed that children with pertussis or experimental animals with *B. pertussis* infection, were found to be hypoglycaemic (Wardlaw and Parton, 1982). Adrenaline is known to cause hyperglycaemia by inhibiting the production of cAMP and subsequent release of insulin by stimulation of α_2-adrenergic receptors. However, in pertussis-vaccinated animals, adrenaline causes hyperinsulinaemia with consequent hypoglycaemia (Katada and Ui, 1976). The molecular mechanism of toxin action was found to be as a result of unresponsiveness at the α_2-adrenergic receptors and stimulation of the β-adrenergic receptor by adrenaline since α- and β-adrenoreceptor antagonists either did not alter or decreased the increased insulin production respectively (Katada and Ui, 1977; Katada and Ui, 1979). The reversal of adrenaline inhibition of cAMP production and consequent insulin secretion was due to the direct modification of inhibitory G-proteins by the S1 subunit of PT (Ui, 1990).

1.4.3.3 Histamine-sensitisation activity

Parfentjev and Goodline (1948) reported that certain strains of mice injected intraperitoneally with pertussis vaccine subsequently became hypersensitive to histamine challenge, resulting in a marked decrease of the lethal dose of histamine (Fishel *et al.*, 1961). PT is primarily responsible for this effect, but, there are reports to suggest that endotoxin preparations from *B. pertussis* and other bacteria can possibly cause histamine sensitisation. Nevertheless, the histamine sensitisation induced by endotoxin is variable and 100% death could not be observed with any dose studied (Pieroni *et al.*, 1966; Munoz and Bergman, 1968a). Apart from histamine sensitisation, PT can also sensitise mice to a variety of substances including serotonin, bradykinin, endotoxin, cold stress and anoxia (Munoz and Bergman, 1968a).

The observed hypersensitivity is dependent on the expression of PT catalytic activity. (Nencioni *et al.*, 1991; Loosmore *et al.*, 1993) However, the precise mechanism of PT in histamine sensitisation is still unknown. Histamine is known to cause vasodilation, promote capillary permeability and bronchoconstriction. Studies indicate that the histamine sensitisation effects are associated with changes in the vascular and not the pulmonary system (de Wildt *et al.*, 1983; Vleeming *et al.*, 2000; Meijeren *et al.*, 2004a). In rats, histamine sensitisation is characterised by decreased diastolic blood pressure and enhanced histamine-induced decrease in blood pressure, and this sensitisation is primarily mediated by histamine H_1 receptor (de Wildt *et al.*, 1983; Vleeming *et al.*, 2000). Consistent with the latter observation, the gene controlling susceptibility to PT-induced hypersensitivity to histamine has recently been identified as the histamine H_1 receptor (Ma *et al.*, 2002). Histamine and other factors including serotonin, bradykinin, cold stress and anoxia, to which PT causes sensitisation, are associated with loss of

blood volume (Munoz and Bergman, 1968; Yong *et al.*, 1993; Vleeming *et al.*, 2000). Moreover mice dying from shock induced by histamine or serotonin were protected by restoring blood volume with physiological saline (Munoz, 1985). Since sensitisation could be duplicated by blocking of β- adrenergic receptors and adrenalectomy, PT has been suggested to impair adrenoceptor function but the role of altered adrenoceptor function in histamine sensitisation is unclear (Fishel *et al.*, 1961; Bergman and Munoz, 1966; Bergman and Munoz, 1968). Others have suggested that the vasoconstricting-regulating mechanism is predominantly involved in PT-induced histamine sensitisation. PT reduced the contractile properties but had no effect on the relaxation properties of small mesenteric resistance arteries following stimulation with various pharmacological agents. Based on these outcomes, it was proposed that PT may produce a condition whereby the contractile properties are unable to counteract the shock syndrome resulting from decreased blood pressure elicited by histamine and may explain why PT can increase the sensitivity to many agents (Meijeren *et al.*, 2004a, b, c). It should be noted that the effect of PT on the relaxation properties were assessed using only histamine or acetylcholine. These studies did not address the effect of PT on the relaxation properties using pharmacological agents for β-adrenoceptor agonists. PT has been shown to inhibit β-adrenoceptor stimulation with salbutamol (de Wildt *et al.*, 1983). In general, this area is not well understood. Nevertheless, the histamine sensitisation induced by PT is used for measuring toxic levels of PT present in vaccines and is discussed in Section 1.6.2

1.4.3.4 Clustering of Chinese Hamster Ovary cells

In CHO cells, PT binds to a 165 kDa membrane glycoprotein which then results in receptor-mediated endocytosis and retrograde transport to at least the Golgi apparatus,

followed by translocation of the PT S1 subunit to the cytosol, and subsequent ADP-ribosylation of a 41 kDa inner membrane protein (Brennan *et al.*, 1988; Burns *et al.*, 1987; el Baya *et al.*, 1997). The modification of G-proteins induces a clustered growth pattern and a change in cell morphology to a more rounded appearance. The onset is slow and requires a minimum of 16 h after the addition of toxin (Hewlett *et al.*, 1983; Gillenius *et al.*, 1985). Although the exact mechanism of toxin action on CHO cells is not clear, it requires the functions of both A- and B-subunits. Fluorescence microscopy studies have indicated cytoskeletal proteins as the target of toxin action since there was a reduction in the percentage of actin filaments remaining in the clustered cells (Scapigliati *et al.*, 1988). Due to the high sensitivity of CHO cells to PT, they are used for monitoring the toxicity levels of PT in vaccines (section 1.6.2.1) and are also used for determining the neutralising capacity of anti-PT serum (Gillenius *et al.*, 1985).

1.4.3.5 T-cell mitogenicity

Stimulation of T- and B- cell proliferation by PT resides in its non-catalytic, binding subunit B-oligomer but the toxin concentration required is much higher (1 μg/ml) in comparison to other *in vivo* or *in vitro* effects of PT (Strnad and Carchman, 1987; Rosoff *et al.*, 1987; Nogimori *et al.*, 1986; Kolb *et al.*, 1990; Nencioni *et al.*, 1990; Loosmore *et al.*, 1993; Tonon *et al.*, 2006). Therefore, the relevant importance of this in natural infection is unclear. Nevertheless, it may be crucial for the adjuvant and protective properties of PT. The mechanisms involved in the activation of T-cells result from cellular signal transduction events induced by the direct interaction of the B-oligomer with plasma membrane proteins and mimic the mitogenic pathway induced by monoclonal antibodies to T-cell receptor complex (TCR-CD3) (Witvliet *et al.*, 1992; Armstrong *et al.*, 1994). PT is unable to activate cell-signalling events related to the

mitogenic pathway in cell lines lacking the TCR-CD3 complex (Rogers *et al.*, 1990; Witvliet *et al.*, 1992). The precise nature of the receptor for activating T-cells is unclear, but 43 kDa and 70 kDa receptors have been identified and sialylated structures appear to be important for toxin recognition (Clark and Armstrong, 1990; Rogers *et al.*, 1990; Armstrong *et al.*, 1994). It has been proposed that the S2 and S3 subunits of PT bind divalently to a multi-subunit receptor complex which in turn interacts with the TCR-CD3 to elicit cellular signalling events (Witvliet *et al.*, 1992; Wong *et al.*, 1996). This results in the activation of the phospholipase C pathway (Rosoff *et al.*, 1987; Strnad and Carchman, 1987; Gray *et al.*, 1989; Thom and Casnellie, 1989) and the subsequent production of IL-2, a molecule that leads to T-cell proliferation (Rosoff *et al.*, 1987; Stanley *et al.*, 1990; Grenier-Brossette *et al.*, 1991).

1.4.3.6 Other activities of PT

PT is well known for its strong adjuvant actions in several immunological systems and include the enhancement of serum antibody response to various antigens, increased cellular immune responses to various protein antigens, contribution to experimental autoimmune encephalomyelitis (EAE), induction of delayed-type hypersensitivity and increased anaphylactic sensitivity (Munoz, 1985; Steinman *et al.*, 1985; Munoz and Peacock, 1990; Roberts *et al.*, 1995; Ryan *et al.*, 1998). Of particular interest is the dual role of PT in protecting against or enhancing experimental autoimmune encephalomyelitis (EAE) (Munoz, 1985; Robbinson *et al.*, 1996). Respiratory tract infections with *B. pertussis* or whole-cell vaccination have been occasionally associated with neurological disorders and encephalopathies (Donnelly *et al.*, 2001). Originally PT was hypothesised to induce EAE by increasing the permeability of the blood-brain barrier. However, recent studies suggest that PT induces EAE by the recruitment of

leucocytes to the central nervous system (CNS) by signalling through Toll-like receptor 4 (TLR-4). Activation of TLR-4 results in the up-regulation of P-selectin on the endothelial surface which facilitates leucocyte access to the CNS and subsequently results in the increased permeability of the blood-brain barrier, thereby providing a mechanism for the breakdown of the blood-brain barrier and induction of the inflammatory response (Kerfoot *et al.*, 2004). Interestingly PT also has protective roles in EAE. This is thought to be due to the differential activities of the A- and B-subunits, but the relative roles of A- and B-subunit activities in enhancing and inhibitory effects on EAE is contradictory (Robbinson *et al.*, 1996; Ben-Nun *et al.*, 1997; Su *et al.*, 2003; Brukener *et al.*, 2003; Kerfoot *et al.*, 2004). It has been suggested that the role of PT in permeabilising the blood-brain barrier may be mediated by the activity of the ADP-ribosyltransferase. By contrast, the B-oligomer mediates an anti-inflammatory effect by competitively blocking leucocyte adherence, and thereby preventing lymphocyte extravasation (Bruckener *et al.*, 2003).

Other functions of PT include its ability to adhere *B. pertussis* to human macrophages and ciliated respiratory epithelial cells, to increase lung permeability and airway oedema, to inhibit HIV replication and virus expression in human macrophages and to inhibit chemotaxis and migration of neutrophils, monocytes/macrophages and lymphocytes (Tuomanen and Weiss, 1985; Relman *et al.*, 1990; Wout *et al.*, 1992; Patterson *et al.*, 1995; Brito *et al.*, 1997; Alfano *et al.*, 2000; Alfano *et al.*, 2001; Garcia *et al.*, 2002) .

1.4.4 Detoxification of PT

PT is considered too toxic for inclusion in vaccine preparations. In all of the acellular pertussis vaccines currently available for human use, PT is detoxified by chemical treatment (PTd) using a variety of chemicals that includes formaldehyde, glutaraldehyde, or both, hydrogen peroxide, or tetranitromethane. An alternative method to detoxify PT involves genetic manipulation (PTg) but these vaccine preparations, although superior to chemically-treated PT vaccine preparations, are not currently available. The genetically inactivated PT (PTg) has substitutions at positions 9 (Arg →Lys) and 129 (Glu →Gly) in the S1 subunit, resulting in a toxoid devoid of ADP-ribosyltransferase activity. The toxoid is also treated with a low concentration of formaldehyde for stabilisation (Pizza *et al.*, 1989; Siber *et al.*, 1991; Edwards *et al.*, 1995; Rappuoli, 1994; Petre *et al.*, 1996).

Different detoxification procedures using various reagents have been shown to result in different amino acid side-chain modifications (Table 1.1) and changes in conformational and epitope binding patterns for the resulting PTds (Burns *et al.*, 1987; Nencioni *et al.*, 1991; Ibsen, 1996). The quality of the resulting toxoid depends on the reagent used and on the extent of chemical modification. The modification of the protein is also affected by factors such as the pH, the availability of the reactive amino acid(s) in the protein, the reactant concentrations and the matrix (Metz *et al.*, 2004). Differential modification of PT by different detoxification methods or subtle changes in the detoxification process itself can have distinct effects on the toxic and immunogenic properties of the resulting PTds (Gupta *et al.*, 1987; Edwards *et al.*, 1995., Metz *et al.*, 2004). Therefore for an effective vaccine, the production of detoxified PT needs to be

carefully monitored in order to achieve a balance between immunological efficacy whilst minimising toxicity levels.

Aldehydes, such as formaldehyde and glutaraldehyde are widely used reagents for the preparation of PTd. In the toxoiding process, formaldehyde reacts primarily with the ε-amino group of lysine residues to form methylol groups followed by a condensation reaction to form a Schiff base (imine), which is rapid and reversible. The imine then cross-links with other amino acids to form a stable methylene bridge (Metz *et al.*, 2004). During the toxoiding process, cross-links can occur within or between different protein molecules (Petre *et al.*, 1996). Glutaraldehyde also reacts in a similar manner to formaldehyde (Table 1.1). It is generally thought that the aldehyde treatment predominantly modifies the B-subunits as evidenced by the formation of higher molecular weight species of the B-oligomer on SDS-PAGE gels and the fact that S1 subunit lacks lysine residues (Nogimori *et al.*, 1986; Nencioni *et al.*, 1991; Fowler *et al.*, 2003). Nevertheless changes in both A- and B-subunits cannot be excluded (Burns *et al.*, 1987; Fowler *et al.*, 2003). In addition, recent studies indicate that methylol groups can be found on the side chains of amino acids other than lysine residues (Metz *et al.*, 2004). To date, the precise nature and location of different chemical modifications on the PT molecule are not known.

Table 1.1. Reactivities of the different chemical reagents used to inactivate PT

Reagent	**Side chain reactivities**	**Reference**
Formalin (formaldehyde) $H_2C=O$	Formaldehyde reacts mainly with ε-amino group of lysine residues but can also react with other amino acids such as cysteine, arginine and histidine to form unstable methylol adducts. The reaction proceeds by a secondary condensation reaction to form an imine (Schiff base) which can then form cross links with other amino acids such as glutamine, asparagine, tryptophan, histidine, arginine, cysteine and tyrosine residues to form a stable methylene bridge.	Fraenkel-Conrat and Olcott, 1948; Tome and Naulet, 1981; Tome *et al.*, 1985; Jiang and Schwendeman, 2000; Metz *et al.*, 2004
Glutaraldehyde $OHC(CH_2)_3CHO$	The reaction of glutaraldehyde is similar to that described for formaldehyde but differs in that it is able to generate multiple stable cross-links without the need for reduction	Habeeb and Hiramoto, 1968; McIntosh, 1992
Hydrogen peroxide H_2O_2	In proteins, it attacks principally via oxidation of the sulphur containing amino acids cysteine and methionine.	Means and Feeney, 1971; Everse *et al.*, 1977
Tetranitromethane $C(NO_2)_4$	Primarily adding nitrate groups to tyrosyl residues. Formation of cross-linkages has been observed in several proteins and is suggested to occur between tyrosine residues	Sokolovsky *et al.*, 1966; Vincent *et al.*, 1970; Everse *et al.*, 1977; Mierzwa *et al.*, 1987; Williams and Lowe, 1971

1.5 Pertussis vaccines

1.5.1 Whole-cell pertussis vaccines (WCVs)

The methods used for the production of WCVs can vary among different manufacturers. In general, WCVs are prepared by growing *B. pertussis* organisms (often more than one strain is used) under conditions that favour the expression of the virulent phase I phenotype and the display of fimbriae 2 and 3. Once the bulk culture is complete, the bacterial cells are harvested, washed to remove substances derived from the medium and suspended in a solution of sodium chloride. The organisms are killed and their toxicity is inactivated by different time-temperature incubations, formalin, thimerosal, long term storage at 2-8^{0}C or some combination of these methods (Griffiths, 1988; Cherry *et al.*, 1988). Currently available WCVs usually combine the killed suspension of *B. pertussis* cells with aluminium-adsorbed diphtheria and tetanus toxoids (DTPw) (European Pharmacopoeia (EP), 2006; WHO recommendations for whole cell vaccines, 2005). In some cases, the *B. pertussis* suspension is adsorbed to either aluminium hydroxide or aluminium phosphate (EP, 2005).

WCVs are used globally and immunisation of infants following approved schedules has shown an efficacy of 80% or more. Despite the success of WCVs, the high reactogenicity has made the use of these vaccines quite controversial. In a study involving 15,752 DTPw recipients and 784 diphtheria and tetanus toxoid (DT) recipients the adverse reactions occurring within 48 h following immunisation were significantly more frequent following DTPw vaccine. Local reactions such as redness, swelling and pain occurred in 37.4, 40.7 and 50.9% respectively of DTPw recipients whereas the percentage of these reactions in DT recipients, was less than 10%. Fever (>38^{0}C), drowsiness, fretfulness, vomiting, anorexia and persistent crying were reported

in 31.5, 31.5, 53.4, 6.2, 20.9 and 3.1% respectively following immunisation with DTPw and again the reaction rate was markedly lower following immunisation with DT (Cody *et al.*, 1981). Apart from these so-called mild reactions, severe side effects such as convulsions, hypotonic-hyporesponsive state, encephalopathy and death have in the past been associated with pertussis vaccination. However, the link between vaccination and some of the severe neurological reactions has never been conclusively proven (Miller *et al.*, 1981; Cherry *et al.*, 1988; Mattoo and Cherry, 2005). Nevertheless WCVs have been shown to induce convulsive activity in mice and was thought to be associated with the presence of PT and LPS. These components are residually present in WCVs but are absent in the acellular pertussis vaccine (ACVs) (Donnelly *et al.*, 2001). The adverse publicity and concern regarding the safety of WCVs decreased vaccine acceptance and, as a consequence, the incidence of pertussis cases increased. The World Health Organisation (WHO) continued to advocate use of WCVs since the benefits of the vaccine outweighed the risk of side effects and concurrently, research on the development of a new (acellular) vaccine devoid of side effects was encouraged.

1.5.2 Acellular pertussis vaccines (ACVs)

The most important finding that led to the development of a 'safer' vaccine was the identification of antigens that conferred protection (Sato *et al.*, 1974 Sato *et al.*, 1984) and since then a variety of ACV formulations has been produced. They differ from each other with regard to bacterial clone, methods of purification and detoxification (section 1.4.4), number and quantity of components, incorporated adjuvants and excipients.

All ACVs currently in use contain PTd either alone or in combination with FHA, PRN or Fims 2 and 3 to give one, two, three, or five component vaccines (Casey and Pichichero, 2005). The bacterial components used in the vaccine are prepared from the

culture supernatant of *B. pertussis* in two ways: co-purification or by individual purification of components. The first approach produces a mixture of antigenic components from *B. pertussis* and involves the precipitation of protective antigens with ammonium sulphate, extraction of soluble proteins with concentrated sodium chloride from the precipitate and then fractionation of the extract by sucrose density gradient centrifugation to obtain a preparation practically free of endotoxin. The second method involves the purification of individual components by successive chromatographic and precipitation steps (Sato *et al.*, 1983; Corbel and Xing, 2004). The bacterial components prepared by either method are then detoxified where required, and blended together to form the one to five component acellular bulk. The combined acellular bulk is adsorbed to aluminium hydroxide or phosphate gel prior to blending it with adsorbed diphtheria and tetanus toxoids to form the triple vaccine (DTaP).

Several clinical trials have been carried out in order to evaluate the safety, immunogenicity, and efficacy of different DTaP vaccines by comparing them with each other and with DTPw products (Klein, 1995; Decker *et al.*, 1995; Edwards *et al.*, 1995; Olin *et al.*, 1997; Cherry, 1997). Although it has been emphasised that differences in methodology and case definition made comparisons between trials difficult, some general comparisons and conclusions were drawn (Cherry, 1997). Overall, the results from the various clinical trials (Klein, 1995; Decker *et al.*, 1995; Edwards *et al.*, 1995; Greco *et al.*, 1996; Gustafsson *et al.*, 1996) showed that DTaP vaccines were associated with less severe and less frequent adverse reactions compared with DTPw vaccine. The DTaP vaccine efficacy improved with an increasing number of antigen components and the best DTaP vaccines showed protective efficacy similar to the best DTPw (Olin *et al.*, 1997;Greco *et al.*, 1996; Gustafsson *et al.*, 1996). The level of antibody necessary for protection is not known but results from the trials indicated that anti-PT, anti-PRN

and anti-Fim antibodies appear to be important (Cherry *et al.*, 1998; Storsaeter *et al.*, 1998).

One finding from clinical trials with serious implications was the ability of formaldehyde treated PTd to revert to toxicity (Ad Hoc Group., 1988; Storsaeter *et al.*, 1988; Storsaeter *et al.*, 1988b; Storsaeter *et al.*, 1990). The ability of formaldehyde treated toxins to revert to toxicity has been previously documented (Akama *et al.*, 1971; Northurup and Chisari, 1972; Sato and Sato, 1988; Cryz *et al.*, 1981). This problem was addressed by Rappuoli and co-workers with the development of PTg, which was also reported to be superior in terms of immunogenicity, but for legal reasons, the product is currently not available. This may not be unethical since its efficacy was found to be similar to that of chemically-detoxified vaccine (Greco *et al.*, 1996).

Despite the lower reactogenicity of DTaP vaccines in comparison to WCV, recent studies have indicated that 1-2% of DTaP booster vaccine recipients display extensive limb swelling at the injection site (Rennels *et al.*, 2000; Yamamoto *et al.*, 2002; Gold *et al.*, 2003; Casey and Pichichero, 2005) which is partly associated with residual activity of PT (Rennels *et al.*, 2000). Hypotonic-hyporesponsive episodes, which are adverse events associated with pertussis vaccines, have also been reported occasionally following immunisation with DTaP vaccine (Braun *et al.*, 1998). Since reversion of PTd to PT can have severe implications in immunisation programmes, regulatory authorities require extensive testing before clinical use to assure the safety of chemically-treated vaccines (1.5.5).

In summary, acellular pertussis vaccine are an innovation that have resulted in less discomfort from vaccination and provided an opportunity to give a late booster dose to

stimulate antibody levels in adolescents and adults which is not possible with WCVs (Pichichero *et al.*, 2006). All licensed acellular pertussis vaccines, that may differ in antigen composition and method of detoxification, have been highly effective in controlling pertussis in infants and children, where vaccine coverage has been high.

1.5.2.1 Acellular-based combination vaccines

In recent years, several other vaccines have been added as a combination with DTaP products to produce multivalent vaccines in order to simplify vaccine administration. The combination of other vaccines is highly advantageous to both manufacturer and recipient since these would induce protection against 4-5 different diseases in one single injection, thereby reducing the distress to the recipient and improving compliance, reduce materials and distribution costs, and prevent the additive exposure to preservatives and stabilisers that can contribute to adverse events (Halsey, 2001). Currently a number of countries have licensed diphtheria, tetanus acellular pertussis based combination vaccines. Three or five component DTaP product are used as a base and combined with some or all of the following vaccines: *Haemophilus influenzae* (Hib) type b polysaccharide (PRP) conjugated to T or D (PRP-T, PRP-D), hepatitis B (HB) and inactivated polio virus (IPV) to form tetra-, penta- and hexavalent vaccines (Vidor *et al.*, 1999; Halsey, 2001). The different types of DTaP-based combination vaccines are shown in Table 1.2 and differ in the amount of pertussis antigens, the type of adjuvant and the presence of other additional antigens.

The decrease in pertussis incidence in developed countries has made it difficult to research on combination vaccines as it is seen as unethical to undertake placebo-controlled clinical trials. Since the disease is uncommon and each vaccine has a known

Table 1.2. Existing and potential acellular pertussis-based combination vaccines. The number of pertussis antigens is shown in brackets.

Commercial name	Composition
Pediacel	DTaP(5)/IPV/Hib
Pediarix	DTaP(3)/Hep B/IPV
Repavax	Tdap(5)/IPV
Hexavac	DTaP(2)/IPV/Hib/Hep B: fully liquid
Infanrix-Hexa	DTaP(3)/Hep B/IPV/Hib (Hib component is lyophilised)
Infanrix-IPV-Hib	DTaP(3)/IPV/Hib
Infanrix-IPV	DTaP(3)/IPV
Infanrix-Hib	DTaP(3)/Hib
Infanrix- Hep B	DTaP(3)/ Hep B
Infanrix	DTaP(3)

record of serological correlates of efficacy, this is used for assessment of combination vaccines. Although this is a satisfactory method for assessing efficacy for most vaccine antigens, it is not appropriate for pertussis antigens since the correlation of specific antibody titres with protection has not been well established. Rather, the immune responses to a combination vaccine are compared to the immune responses elicited by DTaP vaccine with proven efficacy (WHO 1998, EP, 2006).

Results from clinical trials have been promising and have shown that immune responses to combination vaccines including DTaP, HB and IPV are comparable to separately administered vaccines (Vidor *et al.*, 1999; Yeh, 2005; Black and Greenberg, 2005). In sharp contrast, concerns have been raised regarding Hib immunogencity in pertussis-based combination vaccines. A significant reduction in anti-PRP antibody levels was seen following administration of the three component DTaP combination product in comparison with separate administration of Hib and DTaP both clinically and in laboratory studies. Other studies using a different combination containing 5 pertussis components reported no significant effect on Hib immunogenicity (Black and Greenberg, 2005). The reason for this is unclear but it is thought to be due to modulation of Hib in Hib/DTaP combination and possibly results from the interaction of Hib with adjuvant and other vaccine antigens (Mawas *et al.*, 2005, 2006). The clinical relevance of this observation is unclear but may in part account for the increased incidence of Hib cases in England and Wales in recent years (Capiau *et al.*, 2003; McVernon *et al.*, 2003). This unpredictability of immune responses to individual vaccine antigens after incorporating multiple antigens into combination vaccines can have serious implications for the protection against these diseases.

Regarding safety, the reactogenicity of currently available combination vaccines has been shown to be no different from its respective counterparts. Nevertheless, it should be noted that deaths have been observed following administration of the newer pentavalent and hexavalent vaccines (Anonymous, 2004; Yeh, 2005; Zinka *et al.*, 2006). A causal relationship between vaccination and sudden unexpected deaths has not been conclusively proven. Nevertheless, these findings prompt intensified surveillance for unexpected deaths after vaccination. Since pre-licensure Phase I, II and III studies are too small to detect rare or delayed adverse events, post-licensure studies of larger populations for longer periods of time are of paramount importance to assess vaccine safety and effectiveness (von-Kries *et al.*, 2005). The problem with combination vaccines is that it is difficult to pinpoint which component is responsible for a particular adverse event. Currently, the existing guidelines or pharmacopoeial monographs for the laboratory evaluation of DTaP-based combination vaccines are based on experience of tests on individual components (section 1.6). It is assumed that the combination will not display effects not produced by the individual components. However, considering that DTaP vaccines are not without adverse reactions and the complexity of multivalent vaccines, this may not be the case.

1.6 Quality control testing of pertussis vaccines

In general, pertussis vaccines are not considered to be well-defined products since they are derived from, or produced by, a living organism in a batch-wise procedure and, due to the toxoiding procedure, the relationship between physico-chemical or antigenic characteristics and efficacy is not clear. Moreover the negative influence that one vaccine may have on the other in a combination cannot be excluded. For these reasons quality control testing of vaccines is of paramount importance during all stages of

vaccine development for the assurance of product consistency, potency and safety. One of the best examples for demonstrating the need for standardisation of the properties of pertussis vaccines came from the early clinical trial studies with various WCVs where vaccine efficacy ranged from 0 to 100% (Lapin, 1943). Since then it has been learnt that subtle changes in materials, in the process itself or in conditions such as temperature, can result in changes in the final vaccine that can affect its safety, its effectiveness or both. Subsequently, several *in vivo* and *in vitro* control methods have been developed to assure the quality of vaccines. Regulatory agencies, e.g. European Directorate for the Quality of Medicines (EDQM), European Pharmacopoeia (EP), and WHO, generate recommendations and guidelines for the production and control of pertussis vaccines. Vaccine products are assayed for sterility, purity, identity, potency and toxicity and these tests are part of a panel of release assays required by regulatory authorities prior to final release of vaccines for clinical use. This assures that the product is consistently efficacious and at the same time presenting minimal, if any, adverse effects (WHO, 1998; EP, 2005, 2006). Pertussis vaccines are administered to large numbers of healthy people as a prophylactic measure, therefore the assurance of potency and safety of vaccines is essential if effective vaccine immunisation programmes are to be maintained. The current methods used for this purpose have been recently reviewed by Corbel and Xing (2004).

1.6.1 Potency and toxicity tests for WCVs

1.6.1.1 Mouse weight gain test (MWGT)

Currently the control test required by WHO, EP and U.S. for assessing the toxicity of WCV is the mouse weight gain test (WHO, 2005; EP, 2005; FDA, 2006). MWGT is considered to be a general, non-specific test measuring overall toxicity since a number

of *B. pertussis* toxins may affect the weight gain of mice. The exact role of the different *B. pertussis* toxins and their relative contribution to the overall toxicity in vaccine-induced reactions remains elusive. At least three components, DNT, LPS and PT can influence the mouse weight gain. Although the mechanism of toxicity is unclear, the MWGT is detecting mainly endotoxin. DNT is destroyed during the manufacturing process therefore is inactive in vaccine preparation and PT has been shown to interact in this assay in a way that may actually have a suppressive effect on the endotoxin-induced weight loss of mice (Gupta *et al.*, 1988; Horiuchi *et al.*, 1994). Based on this, a refinement of the MWGT is used in some countries since the toxicities of LPS and PT can be differentiated with respect to weight and time (WHO, 2005). Despite these findings, the test has been shown to be useful to some extent since a correlation has been reported between the reactogenicity of pertussis vaccines in the MWGT and the adverse reactions in children (Corbel and Xing, 2004). However, it does not account for the rare cases of severe reactions in children. Separate quantitative assays on each of the biologically-active components may allow for better comparisons with regard to clinical reactivities of pertussis vaccines. Since PT and LPS are thought to largely contribute to the adverse reactions of whole-cell vaccines, regulatory bodies (Section 1.6.2.1) require monitoring of these components using specific methods.

1.6.1.2 Intracerebral mouse protection test

The intracerebral mouse protection (Kendrick) test is the official potency test for WCVs (EP, 2005). The test evaluates the ability of vaccines to protect mice from a lethal intracerebral challenge with a virulent strain of *B. pertussis*. The mechanism of protection by this route is unclear but it is believed that the protection is dependent on alterations of the blood-brain barrier by the presence of active PT in vaccines, thereby

allowing access of antigens, antibodies and immune cells into the brain, and it is the combined effects of minute amounts of PT and other antigens that confer protection (Robinson and Irons., 1983; Munoz and Peacock, 1989; Gupta *et al.*, 1990). The test was established based on the observation of a strong correlation between vaccine efficacy in children and its potency in the Kendrick test (UK Medical Research Council, 1956). However, the artificial mode of protection, technical difficulties and significant intra- and inter- laboratory variation, animal distress and the passing of vaccines of lower efficacy have raised concern regarding its use (Cameron, 1988; van der Ark *et al.*, 1994; Greco *et al.*, 1996; Simondon *et al.*, 1997; van Straaten-van de Kappelle *et al.*, 1997). In order to address these issues a collaborative study was organized by the WHO that required global vaccine manufacturers and national control laboratories to test blinded vaccine samples of high and low potency using the intracerebral mouse protection test. The results of this study showed that the test performed reproducibly with good assay precision. The majority of the participants were able to discriminate between vaccine samples of high and low efficacy (Xing *et al.*, 2001). Nevertheless, some technical problems were highlighted in some of the laboratories that may have led to significant outcomes in terms of passing and failing a vaccine sample if samples with marginal differences in potency had been evaluated. Based on the artificial mode of protection, possible discrepancies in vaccine potencies, animal welfare a better method of assessing potency is required.

1.6.1.3 Alternative potency tests

A serological potency test has been described for whole-cell pertussis vaccine (van der Ark *et al.*, 1994). Although the authors demonstrated that the potency of vaccines determined by this test was significantly similar to that obtained using the intracerebral

mouse protection test, the assay has not been widely accepted as an alternative model for assessing potency for several reasons, even though it can reduce animal distress and the number of mice required by more than 25%. The test is only measuring total antibody binding and there is no assessment of antibody function. Moreover, correlation of serum antibody responses and clinical protection is unclear (Ad Hoc group, 1988; Cherry, 1997). Since the mechanism of protection afforded by whole-cell vaccines involves both cell-mediated and humoral immunity (Mills *et al.*, 1993a; Canthaboo *et al.*, 2001) measuring only total antibody responses may not necessarily represent protection. Thus, the basis of this test is questionable.

The intranasal or aerosol respiratory challenge models have been proposed as suitable alternatives to the Kendrick test. Apart from having the added advantage of assessing both whole-cell and acellular pertussis vaccines, respiratory tract infection of mice has many similarities to human infection: (i) mice are more susceptible to infection when they are young; (ii) localisation of infected bacteria; (iii) similar post-infection physiological changes including lymphocytosis, hyperinsulinaemia and hypoglycaemia and acquired immunity to re-infection (Sato *et al.*, 1980; Redhead *et al.*, 1993; Mills *et al.*, 1998a; Xing *et al.*, 1999; Canthaboo *et al.*, 2000). The aerosol challenge route has been shown to consistently and reproducibly infect the lower respiratory tract, demonstrate a good correlation between bacterial clearance from the lungs after aerosol challenge and the potency of vaccines as estimated by the Kendrick test and with vaccine efficacy in children. However, the complexity and need for specialised aerosol equipment, unlike the intranasal challenge model, has hindered the further validation of this method by an international collaborative study for the acceptance of this method for routine use (Mills *et al.*, 1998b; Canthaboo *et al.*, 2000; Wantanabe *et al.*, 2002; Corbel *et al.*, 2004). On the other hand, a collaborative study was organised by the WHO on the

intranasal challenge model. This method differs in that the *B. pertussis* challenge is carried out by administration of a bacterial suspension directly into the nose using a micropipette and, like the aerosol challenge method, it too was robust and shown to discriminate vaccines of different potencies in a similar performance to vaccines used in clinical trials (Guiso *et al.*, 1999). However, further optimisation of this method is required as no reliable estimates of relative potency could be determined as a linear immunisation dose-response line was not established under the experimental conditions used (Corbel *et al.*, 2004). The respiratory challenge models are not currently part of the release assays as yet but could be anticipated as alternatives in the near future.

There is now evidence indicating that *B. pertussis* can be taken up and survive within macrophages and that clearance involves activated macrophages (Friedman *et al.*, 1992; Xing *et al.*, 1998). Production of reactive nitrogen/oxygen intermediates from activated macrophages is one of the principal mechanisms of macrophage cytotoxicity to invading bacteria. Recent studies showed that immunisation with WCV was associated with the induction of NO synthesis by macrophages and based on this, an *in vitro* nitric oxide induction assay has been developed and reported to form the basis of a potential replacement potency assay (Canthaboo *et al.*, 1999; Xing *et al.*, 2002; Canthaboo *et al.*, 2002). A good correlation was observed between the production of reactive nitrogen/oxygen intermediates and with protective immunity by the aerosol challenge method. Although the data indicate that NO may serve as a useful marker of macrophage activation, like the serological potency assay, measurement of NO production alone may not fully reflect the protective properties of vaccine since antibody-mediated phagocytosis is also a key mechanism for *B. pertussis* killing by phagocytes. Further studies are required to identify the relative roles of the different antigen(s) present in the vaccine for production of reactive nitrogen/oxygen

intermediates and macrophage activation and how this correlates with protection, by immunising mice with various vaccine preparations of different quality.

1.6.2 Potency and toxicity tests for ACVs

The MWGT has proved to be useful for the safety control for pertussis vaccines, but it is not suitable for assessing the toxicity of acellular vaccines since PT/d, which is present in all of the currently available acellular vaccine preparations, may accelerate the weight gain of mice. Therefore, it is considered important to evaluate each of the toxicities separately by specific methods. In ACVs, safety testing involves the monitoring of PT and LPS. These components are considered to be the major players involved in the reactogenicity of WCVs. Other reactogenic *B. pertussis* components are excluded by validation of the manufacturing process. The toxicity tests for residual PT activity in vaccine is the main subject of this thesis and will be discussed below. The tests available for monitoring other *B. pertussis* components have been reviewed by Corbel and Xing (2004).

1.6.2.1 Specific toxicity tests

Currently the HIST is the official safety test for monitoring active PT in ACVs. In HIST, groups of mice are injected intraperitoneally with doses of vaccines, and unvaccinated controls are included. After five days, the mice are injected with histamine solution and the number of mice surviving at 24 h is recorded. A sensitive variant of the test based on the measurement of rectal temperature has been developed in Japan. However has not yet been recommended under the EP or WHO guidelines (Horiuchi *et al.*, 2001). A major problem with HIST is that it is a lethal challenge test, uses large

numbers of animals and large variations in test performance have been observed. The variability arises from mouse strain, age, sex, injection route, challenge route, and environmental factors (Wardlaw and Parton, 1982). Although the assay variability is unacceptable, at present there is no other practical means for detecting active PT in these vaccine preparations. Therefore HIST should be regarded as a priority for replacement. Efforts to develop a new toxicity test based on the mechanism of histamine sensitisation for monitoring PT are still the subject of intense study. As discussed in section 1.4.3.3, the precise mechanism of HIST is unclear but it is known that both A- and B-subunit activities of PT contribute to HIST (Ui *et al.*, 1985; Loosmore *et al.*, 1993). Recently, de Wildts group have shown that PT induces histamine sensitisation probably by interfering with the contractile mechanisms of vascular smooth muscle and has suggested using the contractile mechanisms of the vascular smooth muscle cells of resistance arteries as the basis for a possible alternative to the HIST (Meijeren *et al.*, 2004a, b). However no further developments have been described and it remains to be seen whether this phenomenon is specific for PT and if this approach is able to distinguish toxin from toxoid.

The CHO cell assay (section 1.4.3.4) is an *in vitro* test that may be used as an alternative to the HIST for monitoring active PT in vaccines and was based on the observation that PT specifically causes clustering of CHO cells (EP, 2005; WHO, 1998; Hewlett *et al.*, 1983). In brief, the CHO cells are treated with PT reference or vaccine dilutions. After incubation, the morphological changes are observed under a microscope. The highest dilution of the test vaccine showing total cell clustering represents the titre. The amount of active PT in the test sample can be quantified against a reference preparation of known concentration. This assay, in comparison to HIST, is far superior in terms of sensitivity (Gillenius *et al.*, 1985). Despite its excellent

sensitivity, its use is limited since the assay is not usually suitable for testing the final formulation because of the presence of adjuvant, which causes CHO cell death. However it is useful for monitoring bulk components if they have not been adsorbed to the adjuvant. Another concern regarding the use of the CHO cell assay is the possibility of failing to detect reversion to toxicity after chemical detoxification. It has been shown that CHO cell clustering test failed to detect reversion to toxicity of aldehyde-detoxified PT since the same sample showed significant activity in the HIST. This could be due to the inability to detect activity concealed in aggregates of toxoided PT. The different mechanism of PT action in HIST and CHO is likely to account for the discrepancies (Horiuchi *et al.*, 2001; Kataoka *et al.*, 2002; Corbel *et al.*, 2004).

The lymphocytosis promotion test (section 1.4.3.1) is another *in vivo* assay that can measure active PT in vaccine samples. The assay involves the comparison of peripheral leukocytosis in mice induced by test and reference vaccines. Although this test is useful, it has been reported to be of insufficient sensitivity to demonstrate residual pertussis toxin activity in acellular pertussis vaccines. In addition, the assay is difficult to standardise due to technical problems and this has prevented its use in the routine safety testing of pertussis vaccines (WHO, 1998; Horiuchi *et al.*, 2001).

1.6.2.2 Alternative specific toxicity test

The histamine sensitisation activity is abolished in PTg, in which the enzyme active site has been inactivated by site-directed mutagenesis (Pizza *et al.*, 1989; Rappuoli *et al.*, 1997). Therefore, histamine sensitisation is dependent on the catalytic activity of the A-protomer. A synthetic peptide ($G\alpha_{i3}C20$ peptide) homologous to the carboxyl-terminal 20 amino acid sequence of the α-subunit of the G_{i3}-protein was identified as a good

substrate for PT ADP-ribosylation (Finck-Barbancon and Barbieri, 1995) and, based on this, an enzymatic-HPLC (E-HPLC) coupled assay has been recently developed in which the ADP-ribosylation activity of PT S1 subunit is determined. A synthetic fluorescein-labelled $G\alpha_{i3}C20$ peptide was used as the substrate for the PT A-protomer-catalysed enzymatic transfer of ADP-ribose from NAD to the cysteine moiety of the fluorescent synthetic peptide and reverse-phase HPLC methodology was used to separate and quantify the ADP-ribosylated product (Cyr *et al.*, 2001). Although the ADP-ribosylation activities of native PT preparations using the E-HPLC coupled assay were shown to correlate well with the toxicity observed by HIST (Yuen *et al.*, 2002), the relationship between the ADP-ribosylation activity due to residual PT in vaccines and the *in vivo* HIST has not been investigated. In order to replace the unsatisfactory HIST, a relationship needs to be established between the enzymatic activities in vaccines and their reactivity in HIST. Another concern regarding the use of this *in vitro* assay is that the mechanisms and sites of the different toxoiding processes on the PT molecule have not been defined. Thus PTd, present in pertussis vaccines from different manufacturers, could be modified at different sites of the A-protomer, B-oligomer or both. As mentioned previously, the B-oligomer facilitates host-cell binding and entry of the S1 subunit into the cell and the enzymatic A-protomer catalyses the ADP-ribosylation of G proteins. This dual biological function of the PT molecule is likely to be fully reflected in the in *vivo* HIST but not in the E-HPLC assay which only measures the A-protomer activity. Therefore the E-HPLC method may not fully reflect the *in vivo* toxicity of detoxified PT. Additional assays, to monitor the B-subunit activities, may be needed.

1.6.2.3 Potency test

Due to the unsuitability of the intracerebral mouse protection test for acellular pertussis vaccines and the absence of a clear immune correlate of protection against pertussis, an immunogenicity assay in mice is currently used to monitor the consistency of a test vaccine by comparison with a stable reference vaccine of known clinical efficacy. The immunogenic activity, measured as total antibody response by ELISA, of each antigen claimed to contribute to vaccine efficacy should be within the specification approved by the national control authority, and is based on the immunogenic activity of the corresponding antigen in the reference vaccine (WHO, 1998; EP, 2006). This approach, like the serological potency assay as described for WCVs, measures only total antibody binding and there is no assessment of antibody function. Therefore, assurance of vaccine quality should be made with caution since the test also measures non-functional antibody responses. Moreover, this assay does not take account of cell-mediated immunity. Assessment of cell-mediated immunity can be a useful addition to the immunogenicity test (Mastrantonio *et al.*, 1999; Ausiello *et al.*, 2003).

In Japan, and other Asian countries, a modified Kendrick test has been used for assessing potency of acellular vaccines. This has a similar procedure to the conventional test except that the time of challenge was extended from the original 2 weeks to 3 weeks (Horiuchi *et al.*, 2001). Although this approach has proved to be effective in monitoring potency of pertussis vaccines in Japan, the assay has not been widely accepted due to technical problems and animal welfare concerns. Nevertheless, under the current situation where there is no suitable method for determining potency, the WHO working group on the standardisation and control of pertussis vaccines recommended other countries to start using the modified Kendrick test to determine applicability of the

method since a collaborative study revealed that the method was effective for differentiating immunologically active and inactive preparations (Corbel *et al.*, 2004). Other alternative potency tests that are under investigation include the intranasal and aerosol challenge model and have been discussed previously (1.6.1.3).

1.7 Aims and objectives

The *in vivo* quality control tests play a crucial role in the assessment of the potency and safety of pertussis vaccines and are essential if effective immunisation programmes are to be maintained. However, the assays currently available are far from satisfactory and the continual use of large numbers of animals is not in line with current recommendations to limit the use of animals in the laboratory. Regulatory authorities require safety testing of ACVs in order to confirm the absence of significant residual PT activity. Currently, the HIST is the only practical test for this purpose. However, it is a lethal challenge test and suffers from poor precision. Factors such as mouse strain, age, sex, injection route, challenge route, and environmental factors can affect the reproducibility. Therefore there is an urgent need for a replacement of the HIST. The CHO cell assay is a possible alternative but its use is limited since it cannot be used on final vaccine formulations and may also fail to detect toxic preparations. The E-HPLC coupled assay merits further investigation as an alternative *in vitro* toxicity test but in order to replace the unsatisfactory HIST, the relationship between the ADP-ribosylation activity of residual PT in vaccines and the HIST will need to be established. PT is an AB_5 type bacterial toxin with two functionally distinct domains. The B-oligomer (subunits S2-S5) facilitates host-cell binding and entry of PT into the cell, whereas the enzymatic A-protomer (subunit S1) catalyses ADP-ribosylation of host G-proteins and causes subsequent cell toxicity. A major concern regarding the use of E-HPLC alone is

that it measures only the S1 subunit activity whereas the dual biological functions of the A- and B-subunit activities are both likely to be reflected in the HIST. Therefore, additional assay(s) to monitor the B-subunit activity may be needed to complement the E-HPLC coupled assay. The overall aim of this project was to develop a reliable *in vitro* assay to replace the HIST currently used in the control of pertussis vaccines.

The main objectives were: 1) to further optimise and validate the E-HPLC assay, investigate the effectiveness of the proposed enzymatic assay to determine PT toxicity in different vaccine formulations and to establish its relationship with the *in vivo* HIST; 2) to develop a binding assay for assessment of B-subunit activity of PT in vaccines; 3) to investigate the potential application of the combination of the E-HPLC and binding assays for monitoring active PT in pertussis vaccines and its relationship with the HIST; and 4) to investigate the interaction of PT with CHO cells by comparing the protein expression profile of PT-treated cells with those of non-treated cells using two dimensional gel electrophoresis. Changes in protein expression in CHO cells induced by PT may point the way to alternative toxicity assays.

Chapter 2

Materials and Methods

2.1 Antigens

2.1.1 PT

PT was a freeze-dried in-house reference preparation (NIBSC code 90/518). Each ampoule contained a nominal 20 μg of PT and has been assigned 2100 international unit (IU) of PT activity in terms of the First International Standard for Pertussis Toxin in HIST (Xing *et al.*, 2002).

2.1.2 Pertussis toxoid

Glutaraldehyde (0.5%)-inactivated PT (PTd) was a kind gift of Sanofi Pasteur (France). PTd was prepared for lyophilisation by dialysis, using cellulose membrane with molecular cut off at 12,000 (Sigma-Aldrich, Poole, UK), three times over a period of 24 hours (h) against 10 mM ammonium acetate solution to remove storage buffer (carbonate buffer). After dialysis, the PTd was diluted with 0.5% bovine serum albumin (BSA) solution (1:1 v/v) and aliquots of 500 μl (72.5 μg/ml) were freeze dried and stored at -20^{0}C until use. Protein concentration was determined by UV absorption spectroscopy (2.4.1). Formaldehyde and glutaraldehyde detoxified PT (50 μg/ml) was a kind gift from GSK (Rixensart, Belgium) and was stored at -20^{0}C.

2.1.3 Other Antigens

Diphtheria toxoid (D, 02/176), fimbriae 2 and 3 (Fims 2/3, 85/638), *Haemophilus influenzae* type b capsule conjugated to tetanus toxoid (PRP-T, in-house reference), inactivated polio virus (IPV, in-house reference) and tetanus toxoid (T, 02/232) used in ADP-ribosylation assay or binding assays were all obtained from NIBSC. Filamentous haemagglutinin (FHA, 30 μg/ml) and pertactin (PRN, 50 μg/ml) were kindly donated by GSK.

2.2 Antisera

Mouse anti-FHA monoclonal antibody 1C6 (99/570), mouse anti-*B. pertussis* fimbriae 3 monoclonal antibody (04/156), mouse anti-PT S1 subunit monoclonal antibody 1B7 (99/506), rat anti-D monoclonal antibody (5mg/ml), rat anti-T monoclonal antibody (5 mg/ml), guinea pig anti-PT serum, sheep anti-PT serum (97/572) and sheep anti-PRN serum (97/558) were all obtained from NIBSC (in-house reagents). Monoclonal antibody to fimbriae 2 was kindly supplied by Health Protection Agency (Porton Down, UK).

2.3 Test vaccines

Vaccines used in this study were from three manufacturers with 10 different formulations (A-J). All the formulations used in this study were in combination with D and T ± other antigens such as PRP-T, IPV, FHA, PRN and Fims 2/3. The properties are shown in Table 2.1. Throughout this thesis, for confidential reasons, the exact compositions of the different vaccine formulations used in this study have not been indicated.

2.4 Determination of protein concentration

2.4.1 UV absorption spectroscopy

Concentrations of PTd and FAC ± $[(CH_2)_5]$-$G_{\alpha i3}C20$ were estimated by measuring absorbance (A) near 280 and 495 nm respectively using the Beer-Lambert law:

$$A_{280 \text{ or } 495} = \varepsilon c l$$

Where ε is the molar absorption coefficient (M^{-1} cm^{-1}), l is the path length (cm), and c is

Table 2.1. Pertussis vaccines

Sample	Adjuvant	[PTd] µg/SHD	Method of detoxification
DTaP-A	$Al(OH)_3$	25	FA and GA
DTaP-B	$Al(OH)_3$	25	FA and GA
DTaP-C	$AlPO_4$	20	GA
DTaP-D	$AlPO_4$	20	GA
DTaP-E	$AlPO_4$	20	GA
DTaP-F	$AlPO_4$	20	GA
DTaP-G	$AlPO_4$	2.5	GA
DTaP-H	$AlPO_4$	2.5	GA
DTaP-I (PT-g)	$Al(OH)_3$	5 or 7.5	PT-9K/129G + FA*
DTPw-J	$Al(OH)_3$	N/A	FA

SHD, single human dose (0.5 ml); FA, formaldehyde; GA, glutaraldehyde; * DTaP with genetically inactivated S1 activity of PT was treated with a low concentration of FA (0.035% (w/v) for commercial use (Nencioni *et al.*, 1991).

the protein concentration (M). The UV spectra for PTd (190-370 nm) and FAC ± $(CH_2)_5$-$G_{\alpha i3}$C20 (300-550 nm) were measured at room temperature (RT, ~22^{0}C) using a Cecil 6000 Series spectrophotometer and a matching 1-cm path-length, 1 ml Quartz cuvette (Hellma, Essex, UK). Blank samples for FAC ± [$(CH_2)_5$]-$G_{\alpha i3}$C20 and PTd solutions contained 10 mM ammonium acetate buffer, pH 8.5. The calculated molar extinction coefficient for PT at 280 nm was reported to be 126742 M^{-1} cm^{-1} (Fowler *et al.*, 2003). Concentration of the FAC ± [$(CH_2)_5$]-$G_{\alpha i3}$C20 was calculated using the average molar extinction coefficient of 5-carboxyfluorescein (Aldrich), ε_{492}, 64,400 M^{-1} cm^{-1} (Dr C-T Yuen, personal communication).

2.4.2 Modified Bradford assay

Protein concentrations of cell lysates to be separated by 2-D PAGE (Section 2.10) were determined using a Bradford Protein Assay kit (Bio-Rad) according to the manufacturer's instruction with modification as described by Ramagli and Rodriquez (1985). Standard BSA solutions and each test sample were diluted to 50 µl using the same lysis buffer as that was used to prepare the cell lysates (Section 2.10.1.3). Samples were then individually mixed with 10 µl 0.1 M hydrochloric acid (HCl) and 40 µl distilled water to a final volume of 100 µl in 1cm path-length, 4.5 ml polystyrene cuvettes. Lysis buffer was used as blank. To each replicate sample, 3.5 ml diluted Bradford reagent (1:4 in distilled water) was added and mixed by repeated inversion of sealed cuvettes. After 5 minutes, the absorbance was measured at 595 nm using a Lambda 800 UV spectrophotometer (Perkin Elmer Instruments). A concentration curve corresponding to the absorbance values for BSA ranging from 0-50 µg was performed and the protein concentration of unknown samples were calculated from the slope of the standard-curve using Microsoft Excel.

2.5 Histamine sensitisation test (HIST)

HIST was carried out according to the method described in the WHO guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines (WHO, 1998). NIH strain mice, of 4-5 weeks of age were randomly distributed in groups of 10 per cage. They were inoculated intra-peritoneally (i.p) with each test vaccine at 1 single human dose (SHD, 0.5 ml)/mouse. Three reference groups were injected with 0.5 ml of PT (90/518) in phosphate-buffered saline containing 0.2% gelatin (PBSG) at 5.25 (50 ng) , 1.75 (16.7 ng) and 0.58 IU (5.6 ng) /mouse respectively (Appendix A.1 and A.2). The mice in the negative control group were injected with 0.5 ml PBSG (Appendix A.3). Five days after immunisation the mice were challenged (ip) with 0.5 ml histamine dihydrochloride solution (11.04 mg/ml in PBSG equivalent to 2 mg histamine base per 0.5 ml dose). The number of survivors in each group was recorded after 24 h. The validity criteria for the HIST test were as follows: no deaths in the negative control group after challenge; at 5.25 IU/dose in the PT reference group, > 30% mice show histamine sensitisation; at 1.75 IU/dose in the PT reference group, < 70% mice should show histamine sensitisation. A test result was only taken into consideration if the test met all of the above criteria. Since the HIST is variable, whenever mice in the test group showed signs of reactivity after the histamine challenge, the test was repeated at least once. A batch was regarded as reactive if > 5% mice showed signs of histamine sensitisation from the combined test results.

2.6 High performance liquid chromatography (HPLC)

The HPLC was performed on a titanium PEEK lined Gilson binary pump system fitted with a model 118 UV detector and model 122 fluorescence detector, model 122 (Anachem, Luton, UK). Both operation and data acquisition were controlled and processed by the Gilson Unipoint software.

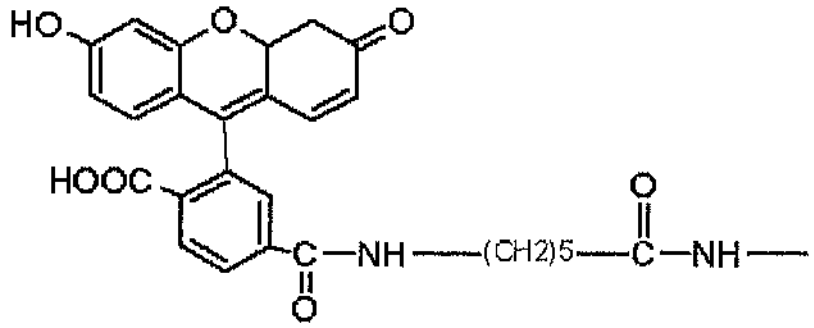
2.7 ADP-ribosylation assay

Adjuvant stock solutions: aluminium hydroxide, alhydrogel (2% $Al(OH)_3$ equivalent to 1.3% Al_2O_3) were from Superfos, Denmark, and aluminium phosphate (0.44mg/ml) was obtained from CSL Limited, Australia. All chemicals and reagents were of analytical grade purchased from Sigma-Aldrich or VWR-BDH, (Poole, UK) and consisted of: adenosine triphosphate (ATP), acetonitrile, ammonium acetate (CH_3COONH_4), chloroform ($CHCl_3$) dithiothreitol (DTT), dimethyl sulphoxide (DMSO), hydrochloric acid (HCl), isopropanol (ISP), lysophosphatidylcholine (LPC), methanol (MeOH), β-nicotinamide adenine dinucleotide (β-NAD), ovalbumin (OVA), phenylmethylsulphonyl fluoride (PMSF), polyoxyethylene (80) sorbitan monolaurate (Tween80), and Trizma®Base. Stock and working preparations of enzyme reaction reagents are shown in Appendix B. The PT enzyme substrate, FAC-$(CH_2)_5$-$G_{\alpha i3}$C20 and FAC-$G_{\alpha i3}$C20 (Fluorescein caproic acid (FAC) tagged $G\alpha_{i3}$C20 peptide, FAC-VFDAVTDVIIKNNLKECGLY-COOH) was custom synthesised by AnaSpec Inc.(San Jose, CA, USA) and was reported to have > 90% purity. FAC-$G_{\alpha i3}$C20 lacks the $(CH_2)_5$ linker between the FAC and peptide (Figure 2.1).

2.7.1 Enzymatic reaction

The enzymatic ADP-ribosylation activity of PT was assayed in duplicate as follows. The PT standard-curve (Appendix B.3) used in each assay was constructed using 13 mM (similar to the amount typically present in vaccines (EP, 2006)) of either $Al(OH)_3$ or $AlPO_4$ as diluents for PT 90/518 according to the characteristics of the test sample (Chapter 3, Section 3.1.2). Vaccine samples, especially in the case of ACVs, generally have high ADP-ribosylation activity. In order to assay them within the PT standard-curve range, they were pre-diluted in OVA (2 mg/ml) solution and used for the ADP-

(a)



(b)

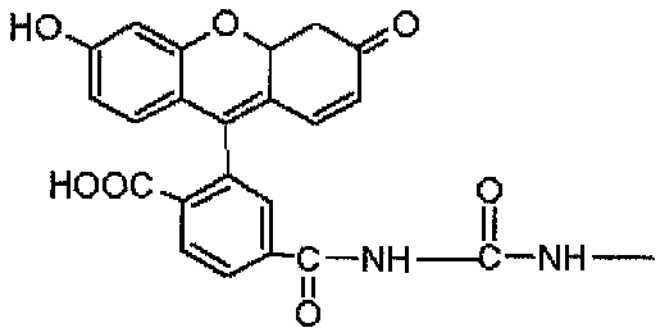


Figure 2.1. Structure of fluorescein tag (FAC TAG) a) with and b) without linker

ribosylation reaction in an identical way to the reference PT. The enzymatic activity in the neat sample (per SHD) was obtained by multiplying by the sample dilution factor.

20 µl of PT 90/518 (Appendix B.3), Biken (in-house positive control vaccine (NIBSC code 00/486), Appendix B.4) and test (vaccine) samples were activated with 5 µl DTT (200 mM, Appendix B.5) for 15 min at RT. Thereafter, 10 µl of ADP-ribosylation reagent (Appendix B.11) was added, mixed and incubated for 30 min at 4^0C. Stock FAC-(CH_2)5-$G_{\alpha i3}$C20 or FAC-$G_{\alpha i3}$C20 (5 µl, Appendix B.12) was added, mixed and incubated at 20^0C for 6 h. The reaction mixture was stopped with 40 µl of DMSO:0.5 M NH_4OH buffer (1:1, v:v) and stored at -20^0C until HPLC analysis. HPLC analysis was carried out within 2 days. The reaction scheme of PT catalysed ADP-ribosylation of FAC-(CH_2)5-$G_{\alpha i3}$C20 peptide substrate is presented in Figure 2.2.

2.7.2 Analysis of ADP-ribosylated product and FAC-$G_{\alpha i3}$C20 by reverse-phase HPLC

ADP-ribosylated product was resolved using a Shodex Asahipak (ODP50-4E) polymeric column (5 µm, 4.6mm x 250mm; Phenomenex, Macclesfield, UK). The HPLC mobile phase was Solvent A, 10 mM CH_3COONH_4, pH 8.5, containing 80% acetonitrile and Solvent B, 10 mM CH_3COONH_4, pH 8.5. Flow rate was 0.8 ml/min and detection was by fluorescence at 495nm (λex) and 515 (λem). Separation was achieved using gradient-elution with solvent B: 75% (0-1 min); 75-70% (1-2 min); 70% (2-6 min); 70-0% (6-8 min); 0% 8-9 min; 75% (9-11 min); and 75% (11-20 min). Prior to the injection of the PT ribosylation reaction mixture (10 µl) on to the column, the frozen solution was thawed, mixed and centrifuged at 10000 rpm (Microcentaur, Sanyo) for 1 min. Ribosylated product and substrates had a retention time of approximately 7.1 and 10.8 min respectively (Chapter 3, Figure 3.2). For the further characterisation of the substrate, separation was achieved using a slower elution-gradient with solvent B: 75%

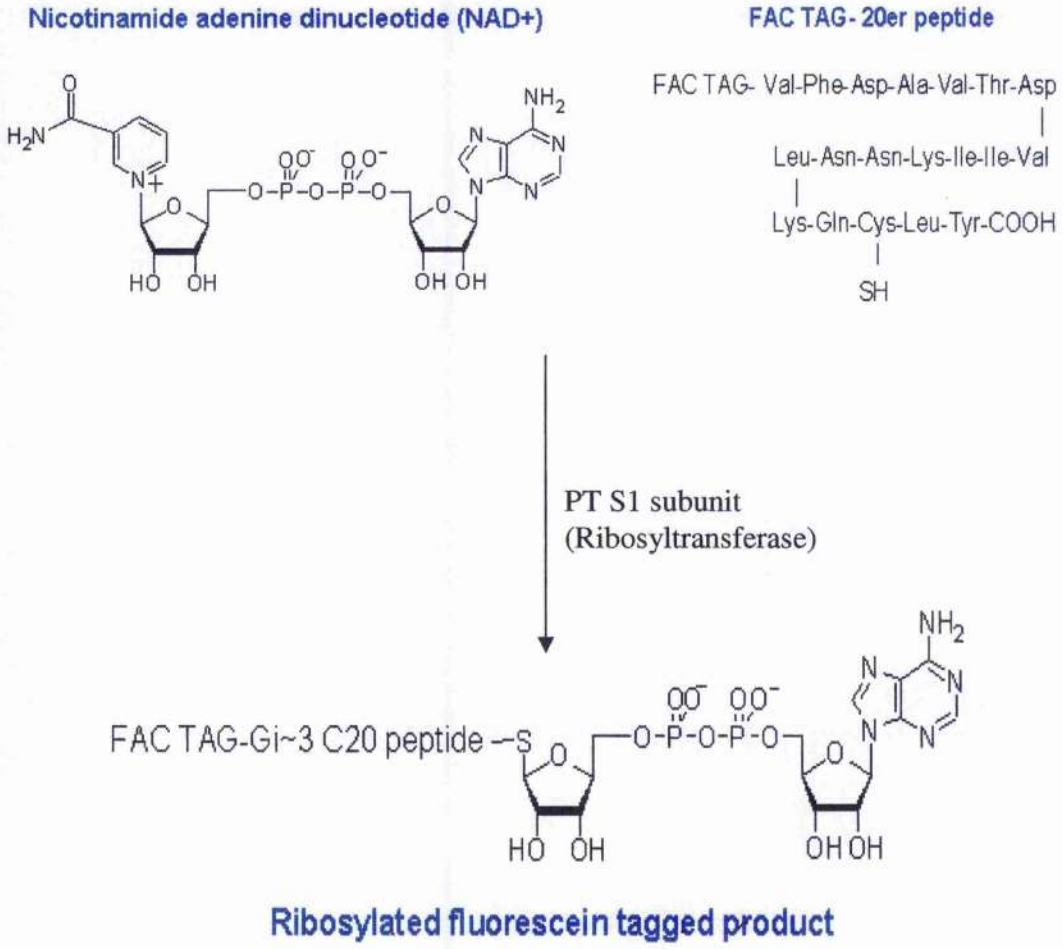


Figure 2.2. Reaction scheme of PT-catalysed ADP-ribosylation of FAC TAG-$G_{i\alpha 3}$ C20 peptide.

(0-3 min); 75-68% (3-4 min); 68-65%; (4-18 min); 65-50% (18-24 min); 0% (24-28 min); and 75% (28-40min).

2.7.3 Data analysis

After HPLC analysis, fluorescence peak area under the ADP-ribosylated product peak was integrated using the Gilson Unipoint software. A PT reference standard-curve constructed with either $Al(OH)_3$ or $AlPO_4$ adjuvant according to the type of adjuvant in the test sample, was used to calculate the enzymatic activity in the test sample by using the linear regression line generated by the Microsoft Excel programme. The enzymatic activity for PT 90/518 was expressed as pM substrate present in the ADP-ribosylated product peak. One pM of fluorescence substrate was deduced to have a fluorescence peak area of 542000. Therefore, the pM substrate was determined by dividing the fluorescence peak area generated by PT 90/518 by 542000. The enzymatic activity for vaccines was defined as: one ADP-ribosylation enzyme unit (E-unit) is equivalent to enzyme activity generated by 1 μg of PT 90/518. Unless otherwise stated, all assays were performed in duplicate and the results presented as means ± standard deviation or CV%.

2.8 Development of a carbohydrate-binding assay for the B-oligomer of pertussis toxin and toxoid

2.8.1 General materials

BSA fraction (grade V), 3'-diaminobenzidine (DAB) tablets, ethylenediaminetetraacetic acid (EDTA), *o*-phenylenediamine dihydrochloride (OPD) tablets, peroxidase labelled: anti-sheep IgG (donkey), anti-mouse IgG (goat), anti-mouse IgM (goat) and anti-rat IgG (goat), polyoxyethylene (20) sorbitan monolaurate (Tween-20), polyoxyethylene (80)

sorbitan monolaurate (Tween-80), tetrabutylammonium cyanoborohydride ($TBACNBH_3$) and tri-sodium citrate were purchased from Sigma-Aldrich or VWR-BDH. All other chemicals were of analytical grade purchased from either Hayman LTD (Essex, UK), Sigma-Aldrich or VWR-BDH. 3-aminopropylbiotinamide (Biotin) and Reacti-Bind Neutravidin coated polystyrene strip plates were purchased from Pierce/Perbio, Northumberland, UK.

2.8.2 Glycoproteins, glycolipids and neoglycolipids

Glycoproteins, glycolipids and negative controls (bovine pancreas ribonuclease A (RNaseA), BSA, 2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) and glucose-ceramide) were all purchased from Sigma-Aldrich-Fluka. Glycoproteins used were: Bovine α_1-acid glycoprotein (bAGP), bovine pancreas ribonuclease B (RNaseB), bovine thyroglobulin-G (bTG), foetal calf serum asialofetuin (Afet), foetal calf serum fetuin (Fet), human α_1-acid glycoprotein (hAGP), human haptoglobin (hHg) and human transferrin (hTf). Glycolipids used were: GM1, GA1, GM2, GM3, GT1b, GD1a and GD1b. Glycoproteins and glycolipids were prepared at 1 mg/ml (w/v) diluted in dH_20 and 1 mg/ml (w/v) diluted in butanol respectively and stored at -20^0C as stock solutions. Neoglycolipids (oligosaccharides.DHPE) consisted of LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP-I, lacto-N-fucopentaose-I; LNFP-II, lacto-N-fucopentaose-II; LNFP-III, lacto-N-fucopentaose-III; SA3LNT, sialyl α2,3-LNT; SA3LNnT, sialyl α2,3-LNnT; SA3LeA5, sialyl LNFP-II; Su3LNT, 3'-sulphated LNT; Su3LeX5, 3'-sulphated LNFP-III; and Su3LeA5, sulphated LNFP-II were kind gifts from Dr. C-T Yuen, The Glycosciences Laboratory, Northwick Park Hospital, Harrow. The structures of glycolipids and neoglycolipids are shown in Table 2.2. The predominant structures found on the glycoproteins tested are shown in Table 2.3.

Table 2.2. Structures of glycolipids (oligosaccharide.cer) and neoglycolipids (oligosaccharide.DHPE)

Glycoconjugates	Structure
GA1	Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1. Cer
GM1	Galβ1,3GalNAcβ1,4(Neu5Acα2,3)Galβ1,4Glcβ1. Cer
GM2	GalNAcβ1,4(Neu5Acα2,3)Galβ1,4Glcβ1. Cer
GM3	Neu5Acα2,3Galβ1,4Glcβ1. Cer
GT1b	Neu5Acα2,3Galβ1,3GalNAcβ1,4(Neu5Acα2,8Neu5Acα2,3)Galβ1,4Glcβ1. Cer
GD1a	Neu5Acα2,3Galβ1,3GalNAcβ1,4(Neu5Acα2,3)Galβ1,4Glcβ1. Cer
GD1b	Galβ1,3GalNAcβ1,4(Neu5Acα2,8Neu5Acα2,3)Galβ1,4Glcβ1. Cer
LNT	Galβ1,3GlcNAcβ1,3Galβ1,4Glc. DHPE
LNnT	Galβ1,4GlcNAcβ1,3Galβ1,4Glc.DHPE
LNFP-I	Fucα1,2Galβ1,3GlcNAcβ1,3Galβ1,4Glc.DHPE
LNFP-II	Galβ1,3(Fucα1,4)GlcNAcβ1,3Galβ1,4Glc.DHPE
LNFP-III	Galα1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glc.DHPE
SA3LNT	Neu5Acα2,3Galβ1,3GlcNAcβ1,3Galβ1,4Glc.DHPE
SA3LNnT	Neu5Acα2,3Galβ1,4GlcNAcβ1,3Galβ1,4Glc.DHPE
SA3LeA5	Neu5Acα2,3Galβ1,3(Fucα1,4)GlcNAcβ1,3Galβ1,4Glc.DHPE
Su3LNT	SO_3-3Galβ1,3GlcNAcβ1,3Galβ1,4Glc.DHPE
Su3LeA5	SO_3-3Galβ1,3(Fucα1,4)GlcNAcβ1,3Galβ1,4Glc.DHPE
Su3LeX5	SO_3-3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glc.DHPE
DHPE	$C_{37}H_{78}NO_6P$*

*2- Dihexadecyl-sn-glycero-3-phosphoethanolamine. Cer, ceramide; Glc, glucose; Gal, galactose; GlcNac, N-acetyl glucosamine; GalNac, N-acetyl galactosamine; Neu5Ac, N-acetyl neuraminic acid, commonly known as sialic acid; Fuc, fucose. SO_3, sulphate.

Table 2.3 *N*-glycan structures predominantly found on glycoproteins

Glycoprotein	*N*-glycans	Reference
bAGP	A2 (contains *N*-glycolyl and *N*-acetyl sialic acids)	Nakano *et al.*, 2004
RNaseB	High mannose structures (Man 5-9)	Joao *et al.*, 1993; Rudd *et al.*, 1995
bTG	Complex (contain sialic acid, fucose and galactose residues) and high mannose structures	Rawitch *et al.*, 1993
Afet	Prepared by removal of sialic acid from fetuin, yielding neutral complex type structures NA2, NA3	
Fet	A2 and A3	Takasaki *et al.*, 1986
hAGP	A2, A3 and A4	Fournet *et al.*, 1978; Yoshima *et al.*, 1981
hHg	A2, A3	Sieczkowska and Olczak., 2001
hTf	A2	Charlwood *et al.*, 1998

2.8.3 Oligosacccharides

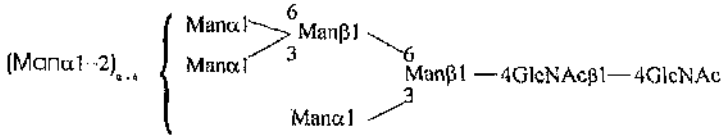
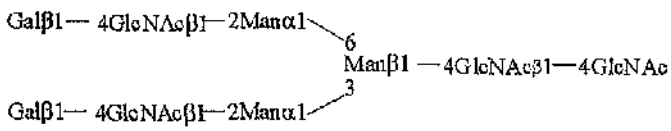
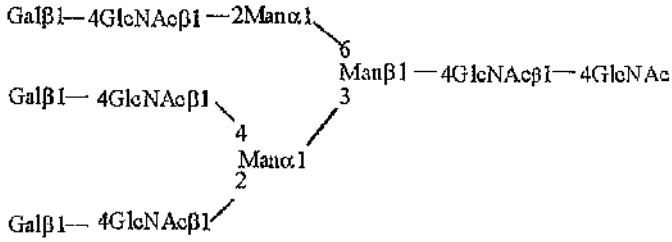
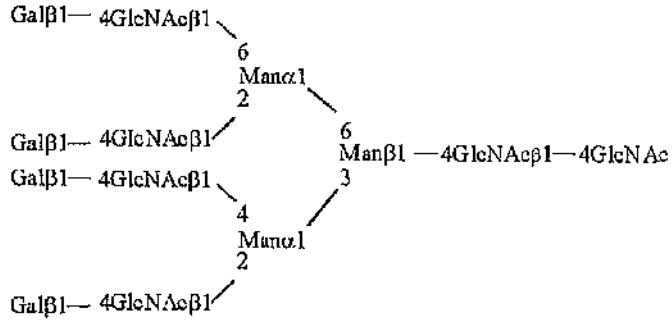
N-glycans: mono sialylated biantennary (A1); di-sialylated biantennary (A2); tri-sialylated triantennary (A3); tri-sialylated triantennary with core fucose (A3F); tetra-sialylated tetraantennary (A4); tetra-sialylated tetraantennary with core fucose (A4F); asialo-biantennary (NA2); asialo-triantennary (NA3); asialo-tetraantennary (NA4) were either purchased from Dextra Laboratories (Reading, UK) or from Ludger Glycotechnology (Oxford, UK) or prepared in-house from glycoproteins (hTf, bovine Fet and hAGP (Sigma-Aldrich, Poole, UK) according to previously published procedures (Yuen *et al.*, 2002). Other *N*-glycans were prepared in-house by Dr. C-T Yuen and consisted of *N*-glycolyl (Ngc) and *N*-acetyl (NAc) sialylated *N*-glycans: A2-1, di-sialylated (1 Ngc, 1NAc) biantennary; A2-2, di-sialylated (2Ngc) biantennary, A2-3, tri-sialylated (1Ngc, 2NAc) biantennary; A2-4, tri-sialylated (3Ngc) biantennary; A3-1, mixture of tri-sialylated (1Ngc, 2NAc) and tri-sialylated (2Ngc, 1NAc) triantennary and were prepared from bAGP. High mannose type *N*-glycans were prepared from RNaseB (Yuen *et al.*, 2002). Galactose (Gal), maltopentaose (Glc5), lactose (Lac) and sialyl α2-3/6-lactose (SaLac) were purchased from Sigma-Aldrich, Poole, UK. The structures and monosaccharide compositions of these glycans are listed in Tables 2.4 and 2.5. Since native oligosaccharides do not adhere directly to microwells, oligosaccharide structures were therefore derivatised with an amino-biotin derivative (section 2.8.4) and immobilised to neutravidin-coated wells.

2.8.4 Preparation of biotinylated (BTN) glycans and glycoproteins

2.8.4.1 Conjugation of oligosaccharides or glycoproteins to biotin

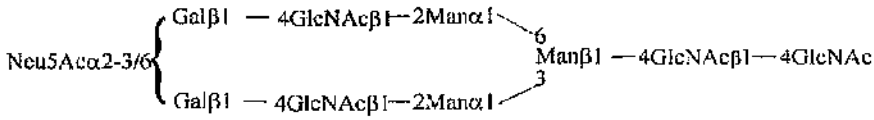
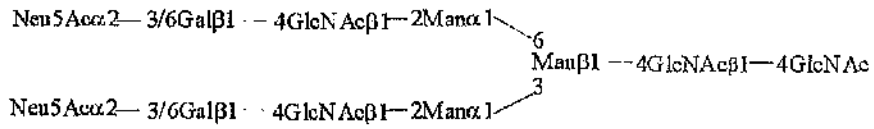
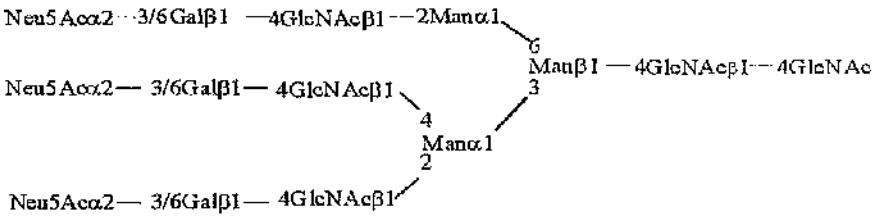
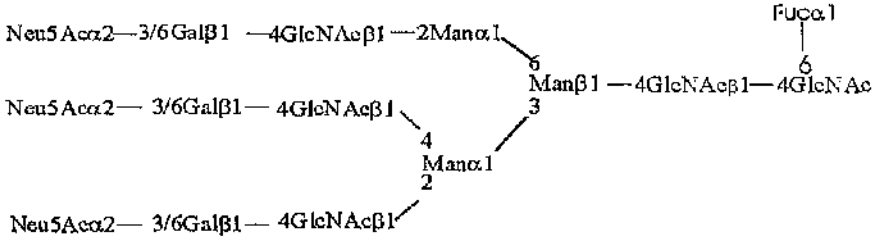
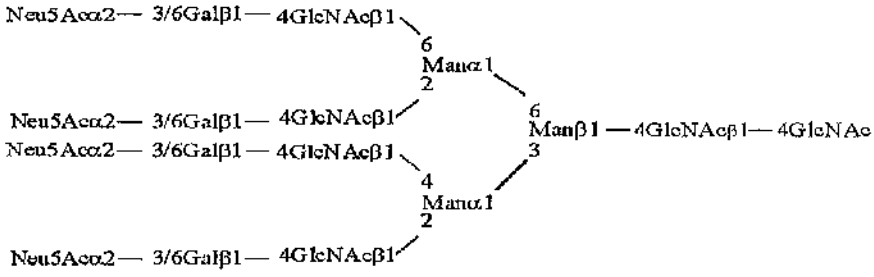
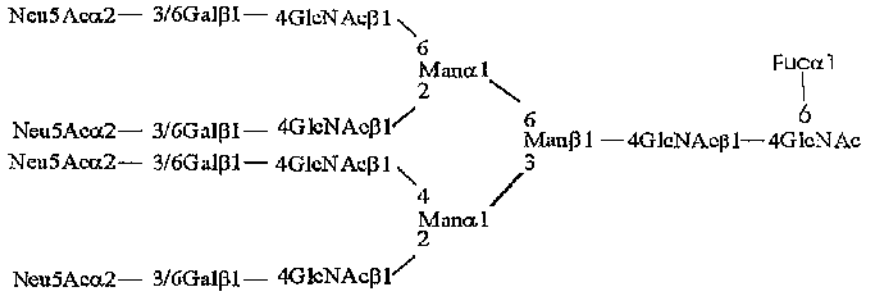
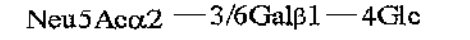
Oligosaccharides (Tables 2.4 and 2.5) were conjugated to *N*-(3-aminopropyl) biotinamide by reductive amination (Figure 2.3) as described (Yuen *et al*, 2000 and

Table 2.4. Structures of neutral oligosaccharides*

Oligosaccharide	Structures
Man5-9	
NA2	
NA3	
NA4	
Glc5	Glcα1—4Glcα1—4Glcα1—4Glcα1—4Glc
Lac	Galβ1—4Glc

* For the binding experiments oligosaccharides were assayed as biotinylated oligosaccharides. Man5-9, oligomannose 5-9 type *N*-linked oligosaccharides; GlcNAc, N-acetyl glucosamine; Man, mannose; Gal, galactose; Glc, glucose.

Table 2.5 Structures of acidic oligosaccharides*

Oligosaccharide	Structures
A1	
A2	
A3	
A3F	
A4	
A4F	
SaLac	

* For the binding experiments oligosaccharides were assayed as biotinylated oligosaccarides. A2-1 to A2-4 and A3-1 differs only in the sialylated structures present but have the same structure of A2 and A3 structures respectively (section 2.9.2). GlcNAc, N-acetyl glucosamine; Man, mannose; Gal, galactose; Neu5Ac, N-acetyl neuraminic acid, commonly known as sialic acid; Glc, glucose.

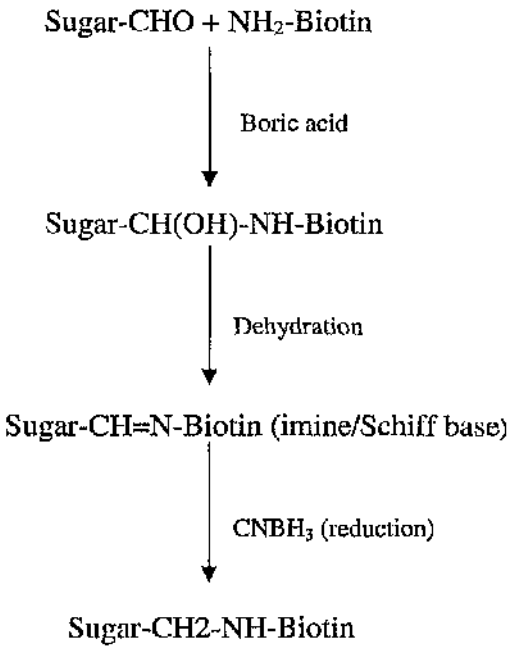


Figure 2.3. Reaction scheme for formation of biotinylated oligosaccharides. In brief, the boric acid prepares the aldehyde group of the reducing end of the oligosaccharide (carbonyl carbon) for the nucleophilic attack by the amine group of the biotin. This forms an intermediate product with a hydroxyl group and a new C-N bond (hemi-aminal). The acidic medium aids in the protonation of the hydroxyl intermediate to allow water to leave, forming the partially stable Schiff base. The Schiff base imine group is chemically reduced to give a stable derivatised glycan using tetrabutylammonium cyanoborohydride.

Feizi *et al*, 1994) with slight modifications. Lyophilised oligosaccharides (~10-100 nM) in 1ml- polypropylene v-vials were dissolved in boric acid (10 µl, 0.5 M in 50% ethanol) and added to *N*-(3-aminopropyl) biotinamide (80 µl, 10 mM in MeOH) and the mixture was gently vortexed. Then, freshly prepared tetrabutylammonium cyanoborohydride ($TBACNBH_3$, 10 µl, 1 M in MeOH) was added, mixed and the reaction mixture capped tightly and incubated on a heating block at 60^0C for 3 h. When making $TBACNBH_3$ solution, it should be noted that 1 mg of $TBACNBH_3$ solid would take up ~1 µl volume when dissolved). After incubation, the cap was opened and the reaction mixture was allowed to dry at 60^0C by evaporation (~40 min) in a fume cupboard. The derivatized (biotinylated) oligosaccharides (BTN-oligosaccharides) were re-suspended in distilled water (200 µl). To remove excess $TBACNBH_3$ 300 µl of ethyl acetate was added to the solution, vortexed to mix, centrifuged (1 min at 10,000rpm, Minispin Eppendorf) and the upper organic layer was removed (biotinylated oligosaccharide is present in the lower aqueous phase). This procedure was repeated five times in total. After ethyl acetate extraction, biotinylated oligosaccharides in the lower aqueous phase were lyophilised. These partially purified BTN-oligosaccharides were then subjected to further purification by HPLC on a TSK Amide-80 column (Section 2.8.4.2). Biotinylation of fetuin and ribonuclease B glycoproteins was performed using biotin N-hydroxysuccinimide according to the manufacturer's instructions (Pierce, UK).

2.8.4.2 Purification of biotinylated oligosaccharides by normal phase HPLC

Biotinylated oligosaccharides were purified by normal-phase HPLC on an TSK Amide-80 column (Anachem; 5 µm, 4.6 x 250 mm) as described previously (Yuen *et al.*, 2002) with minor modification of the solvents and elution gradient conditions, to remove unconjugated biotin and other carbohydrate impurities. Chromatographic separation was

achieved using gradient elution over a period of 1 h at a flow rate of 1ml/min. Solvents A and B consisted of 80% and 20% acetonitrile, respectively, and both contained 5 mM trifluoroacetic acid (TFA) and 10 mM CH_3COONH_4, pH 4.2 (final pH ~2.5). The elution gradient profile of solvent B was as follows: 30% (0-1 min) 30-45% (1- 40 min), 45-80% (40-48 min), 80-30% (49-51 min) and 30% (51-60 min). The system was calibrated using 4-ABA- labelled glc oligomers to create a dextran ladder as previously described (Yuen *et al.*, 2002). Detection of 4-ABA-labelled glucose references was by fluorescence (λex, 296nm; λem, 359nm). Biotinylated oligosaccharides were detected by UV absorption at 223 nm. Lyophilised biotinylated oligosaccharides (section 2.8.4.1) were reconstituted to a total volume of 100 µl in a two step procedure with starting solvent, 70% solvent A and 30% solvent B (first step, 70 µl; second step, 30 µl and pooled) prior to injection into the HPLC system. After each step the samples were vortexed and centrifuged at 10000 rpm (Minispin Eppendorf) for 10-20 seconds to maximise recovery of the oligosaccharides. HPLC fractions were isolated on elution and lyophilised.

2.8.4.3 Identification of biotinylated oligosaccharides

All the structures of the glycans prepared in-house and derivatised oligosaccharides and 4-aminobenzoic acid derivatives were confirmed by mass using a matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometer (MS) (Bruker Autoflex; Coventry, UK) operated in the positive ion reflection mode with 2,5 dihydroxybenzoic acid (Sigma-Aldrich, UK) as matrix. Structures were also confirmed from their relative elution times compared with authentic commercial standards (when available) by TSK Amide-80 HPLC. The lyophilised biotinylated *N*-glycan fractions (post HPLC purification) were re-dissolved in 100 µl distilled water. 1 µl of sample (~10-100 pM) was mixed with 1 µl of matrix (2,5-dihydroxybenzoic acid (20 mg/ml in

0.05% TFA, 50% ethanol)) and 1 µl of this mixture was spotted on target and allowed to dry at ambient temp and subjected to MALDI-TOF MS. The results are presented in Chapter 3, Table 3.3.

2.8.4.4 Quantification of biotinylated oligosaccharides by orcinol staining

Quantification of biotinylated oligosaccharides was carried out on normal aluminium-backed TLC plates (Merck) using an orcinol sulphuric acid staining procedure (Dubois *et al.*, 1956 and Feizi *et al.*, 1994). 1 µl of known amount of galactose (0.05-0.5 mg/ml) and 1 µl of unknown amounts of biotinylated oligosaccharides were spotted on TLC plates and allowed to dry. 3,5-dihydrotoluene-monohydrate (Orcinol, Sigma-Aldrich) reagent (Appendix C.1) was sprayed evenly just until the plates appeared slightly wet and thereafter heated in a vented oven at 120^0C for about 1 min or until the violet colour of hexose was maximal. Quantification was performed after image acquisition using a ProtoCOL colony counter (Synbiosis, Cambridge, UK) fitted with a CCD camera (Sony DFW/SX900) and using GeneTools analysis software (SynGene, Cambridge, UK). The amount of hexose present in the biotinylated oligosaccharides was derived from the galactose standard-curve. Total amount of biotinylated oligosaccharide was calculated from the relative percentage of hexose present in the oligosaccharide structure. For example, the amount of hexose present in biotinylated A3 was found to be 0.4 mg/ml, which only accounts for 30.7% of hexose content in A3. Then the total amount of oligosaccharide would be 0.4 x 3.26 (100%) to give amount of oligosaccharides per ml. The percentages of hexose present in the oligosaccharide structures tested are shown in Chapter 3, Table 3.3.

2.8.5 Investigation of PT binding to glycolipids using DOT blot method

1 µl of stock preparations of glycolipids (1 mg/ml) were spotted directly on nitrocellulose membrane (Hybond-C super membrane, RPN203G 91448, Bio-Rad) allowed to dry and then blocked with 3% BSA diluted in PBS for 2-3 days at 4^0C. After blocking, the membrane was washed four times with PBS and incubated with 20 ml of 1 µg/ml PT (diluted in PBS) for 2 h at 37^0C with shaking (20 revs/min Stuart shaking incubator: S150, Stuart Scientific, UK). Following washing of the membrane with PBS, the blot was then incubated with 20 ml of sheep anti-PT serum (1/10,000 diluted in PBST) for 2 h at 37^0C with gentle agitation (20 revs/min). The membrane was then washed three times with PBST and subsequently incubated with 20 ml of peroxidase-labelled anti-sheep IgG (1/1000 diluted in PBST) for 2 h at 37^0C with shaking (20 revs/min). The membrane was washed three times with PBST and developed with 15 ml DAB solution, prepared according to manufacturer's instruction, for about 5-10 min at RT shaking (20 revs/min). The reaction was stopped by washing with distilled water and blotted dry. Dot blot images were captured using a white light trans-illuminator connected to an image acquisition and analysis software package (LabWorks, www.uvp.com).

2.8.6 Investigation of PT binding to neoglycolipids by neoglycolipid-capture ELISA

Neoglycolipids were coated on Immulon 4 plastic microwells (Thermo Labsystems) as described by Green *et al.*, 1992. Binding of PT was performed as described for glycoprotein-capture ELISA (Section 2.8.9).

2.8.7 Screening of glycoproteins as ligands for PT binding

A total of 8 different glycoproteins were investigated for their suitability as a ligand for PT binding and the non-glycosylated proteins RNaseA and BSA were used as negative controls. The procedure was performed as follows: Nunc maxisorp-96 well ELISA plates (VWR-BDH, UK) were coated with 1 µg of glycoprotein (100 µl per well in carbonate buffer (pH 9.5) containing 0.035 M sodium hydrogen carbonate, 0.015 M sodium bicarbonate and 7.4 mM sodium azide) at RT (~22°C) overnight (O/N). Plates were blocked with 3% BSA in PBS containing 0.05% v/v Tween-20 (blocking buffer, PBST, 100 µl per well) for 1 h followed by incubation with PT at 25 ng/well (positive control, 100 µl per well) or test samples at RT for 2 h. Serial dilution across the plate was performed using the blocking buffer as the diluent. Negative control wells were incubated with blocking buffer. Thereafter, 100 µl of sheep anti-PT serum (NIBSC, 97/572, 1:50,000 in blocking buffer) was added and incubated at RT for 1 h 30 min, followed by incubation for 1 h 30 min with 100 µl per well of horseradish peroxidase-labelled anti-sheep IgG (1:1000 in blocking buffer). Optimal concentrations of both primary and secondary antibodies were determined using checkerboard titrations. The colour development was carried out according to manufacturer's instructions by adding 100 µl OPD substrate solution to all wells and incubation in the dark at RT (15-20 min), followed by the addition of 50 µl of 3 M HCl to stop the reaction. The plates were read immediately at 492 nm using a Multiskan MS plate reader running on Genesis software for Windows (Labsystems, UK).

2.8.8 Screening of oligosaccharides as ligands for PT binding

The binding activity of PT towards a panel of 17 different oligosaccharides was

examined. The procedure was similar to that described for screening of glycoproteins, except for the following changes: Neutravidin plates (pre-blocked in blocker BSA, Pierce, UK) were used and they were coated by incubating with biotinylated glycans or biotinylated glycoproteins (30 pM/200 µl) for 2 h at room temperature. The capacity of biotin binding by the plates, according to the manufacturer is about 25 pM using 200 µl incubation volumes. After washing, wells were incubated with PT 90/518 at 500 ng/well (200 µl per well) for 2 h at RT. Following incubation for 1.5 h with 200 µl per well of sheep anti-PT serum (1:100,000 in blocking buffer), the binding was quantified by incubating ELISA plates for 1.5 h with 200 µl per well of horseradish-peroxidase-labelled anti-sheep IgG (1:2000 in blocking buffer) and subsequent colour development with OPD substrate solution (200 µl per well) in the dark (30 min). Colour development was stopped by adding 3 M HCl (50 µl per well) and the developed colour was read immediately using a Multiskan MS plate reader at wavelength 492 nm. Optimal concentrations of both primary and secondary antibodies were determined using checkerboard titrations.

2.8.9 Determination of binding activity of PT and PTd using glycoprotein- or oligosaccharide-capture ELISA

The binding activities of PT and PTd were assessed using glycoprotein or oligosaccharide-capture ELISA described above with the following exceptions for oligosaccharide-capture ELISA: sheep anti-PT serum was used at 1:80,000; horseradish peroxidase-labelled anti-sheep IgG was used at 1:2000; and serial dilution of PT and vaccine test sample (DTaP A-H) using desorption buffer (section 2.8.8). The blocking buffer was used as negative control and PT was used as reference. The binding activities in the test samples were either expressed as potency relative to the reference vaccine by

parallel line assay using the linear portions of the titration curves for the test sample and the reference preparation (Finney, 1978) or the PT reference standard-curve was used to calculate the binding activity in test sample by using the linear regression line generated by the Microsoft Excel programme. The binding activity for the latter case is defined as: one binding unit (B-unit) is equivalent to binding activity generated by 1 μg of PT 90/518. For investigation of the effect of other vaccine components on the binding assay, the optical density (OD) values of negative controls were subtracted from the OD values of the reference toxin (PT) and test sample and the binding activity in the test sample was presented as % of the OD values generated by binding of PT at 31.25 ng/well (100%).

2.8.10 Desorption of antigens from aluminium adjuvant suspensions

Three different desorption buffer systems: tri-sodium citrate (citrate; 100 mg/ml diluted in PBS) or EDTA (3 mM diluted in 0.25 M Na_2HPO_4) or Tween-20-EDTA (0.2% Tween-20 and 0.2% EDTA) were used to investigate their efficiency in eluting the antigen from the aluminium adjuvants. Equal volumes of vaccine and desorption buffer were mixed and incubated O/N at 37^0C and with gentle shaking (150 revs/min, Stuart Scientific, S150 UK). Aluminium adjuvants were then removed by centrifugation at 13200 revs/min (Eppendorf 5415D) for 10 min at RT and the supernatant was used immediately for the binding assay.

2.9 Exploring the potential applications of ADP-ribosylation assay and carbohydrate binding assay

2.9.1 Comparison of different formulations

Vaccine samples were treated with and without Tween-80 and kept at 4^0C for two weeks. PBS (Appendix A.3) was used instead of Tween-80 as control. Histamine sensitisation, ADP-ribosylation and fetuin binding activities were assessed according to the method described in sections 2.5, 2.7 and 2.8.7 respectively. The monoclonal antibody-capture ELISA experiment for PT subunits S1 and S2/3 is described hereafter. ELISA plates were coated with 100 µl of 1/100 monoclonal anti-PT S1 (NIBSC code 99/506) and 1/100 monoclonal anti-PT S2/3 (NIBSC code 99/534) diluted in carbonate coating buffer. Plates were left overnight in a humid box at 37^0C. After washing three times with PBS (Appendix A.3 (i)) containing 0.05% Tween-20, plates were blocked with 100 µl/well of 10% FCS diluted in PBST. For preparation of vaccine samples, DTaP-A and DTaP-C were desorbed using tri-sodium citrate as described in Section 2.8.10 and the supernatant was used for the assay. Two-fold serial dilutions of the vaccine sample and PT 90/518 (positive control, 1000-8 ng/ml) was carried out in ELISA plates and after loading, plates were incubated for 2 h at RT (~22^0C). After washing the plates three times with PBS, they were incubated with 100 µl of guinea pig anti-PT serum (1:1000 diluted in blocking buffer) and incubated for 2 h at RT. Thereafter, following another washing step, 100 µl of horseradish peroxidase-labelled anti-guinea pig IgG (1:2000 diluted in blocking buffer) was added and incubated for a further 2 h at RT. The colour development was carried out as described in Section 2.8.7. The plates were read immediately at 492 nm using a Multiskan MS plate reader running on Genesis software for Windows (Labsystems, UK). The binding activities in the

DTaP products treated with Tween-80 were expressed as potency relative to the corresponding reference DTaP products without Tween-80 by parallel line assay as described in Section 2.8.9.

2.9.2 Preliminary interpretation of the relationship between the *in vitro* and *in vivo* tests of PT toxicity

The following mathematical equation [1] was used according to the method described by the Heinemann working group (Chapter 2, Solving equations and inequalities, 2002) to explore the possible relationship between histamine sensitisation, enzymatic and binding activities in this study

$$\text{Death} = A\,(H \times B) + C \qquad [1]$$

where A is an associated parameter for enzymatic-HPLC (H) and binding (B) activity and C is a constant parameter. The suitability of this model was assessed by fitting the model to the experimental data obtained in each of the three assays using PT 90/518. Using the mean values for histamine sensitisation (% death), enzymatic (pM substrate) and binding activities (OD values) for PT at 16.7 and 50 ng PT 90/518, two equations could be generated and are as follows:

16.7 ng PT $\qquad 23 = A \times (25.11 \times 0.20) + C \qquad [2]$

50 ng PT $\qquad 70 = A \times (75.64 \times 0.42) + C \qquad [3]$

The A value was calculated by subtracting equation 2 from 3 (Heinemann writing group, 2002). The C value was calculated by substituting the A value into equation 2 or

3. The calculated A and C values were inserted into equation [1] and the mathematical model to correlate death with enzymatic and binding activity is shown in the equation below,

$$\text{Death} = 1.77\ (H \times B) + 14.4 \qquad [4]$$

2.10 Proteomic analysis of PT-induced CHO cell clustering

A 2-D gel electrophoresis method was used for the proteomics study and involves: sample preparation, 2-D gel electrophoresis (both analytical and preparative), image analysis and mass spectrometry for protein identification and this work was carried out in collaboration with Dr. J. Wheeler, Laboratory of Molecular Structure, NIBSC. The aim was to investigate the proteomic changes induced by PT in CHO cells but due to limited time, training could not be given on mass spectrometry, therefore protein identification was kindly carried out by Dr. J. Wheeler.

All reagents and equipments used, unless specified, were purchased from Amersham Biosciences, Buckinghamshire, UK.

2.10.1 Preparation of CHO cells

2.10.1.1 Cell culture

CHO K1 cells (Cat No. 03-402-83, ECACC No.8505/005) were maintained in RPMI 1640 with glutamine and supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), 100 units/ml penicillin and 100 µg/ml streptomycin (tissue culture

medium, all purchased from Invitrogen, Paisley, UK). Cell cultures were maintained in a monolayer culture at 37^0C in humidified air with 5% $C0_2$.

2.10.1.2 Treatment of CHO cells with PT

CHO cell clustering assay was carried out as described by Gillenius *et al.* (1985). Confluent flasks of CHO cells were aspirated, washed twice with sterile PBS (Appendix A.3 (i)) and the cells were detached by incubating with 5 ml of 0.25% trypsin solution (Life technologies, UK) for approximately 3 min at RT at which point the solution was removed, and then the flask was incubated for 5 min at 37^0C. Detached cells were re-suspended in tissue culture medium and adjusted to a concentration of 2 x 10^4 cells/ml using Trypan Blue dye exclusion. 50 ml of cell suspension (1 x 10^6 cells) were transferred to 75 cm^2 tissue culture flask followed by the addition of 6.7 ml of tissue culture medium (negative control) or PT 90/518 at 64 ng/ml (diluted in tissue culture medium) and incubated for 48 hrs at 37^0C.

2.10.1.3 Sample preparation for gel electrophoresis

The treated and untreated control cells were detached, re-suspended in tissue culture medium and cell count and viability were obtained using Trypan Blue exclusion. 5 x 10^6 cells were pelleted by centrifugation at 1200 rpm (Legend RT, Sorvall) for 5 min and re-suspended in ice-cold PBS. Centrifugation was carried out at 4^0C. This washing procedure was repeated three times to remove potential contaminants from culture media. The final cell pellet was lysed in 40 µl of lysis buffer (Appendix D.1) using three different methods:

1. Normal lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 10 mM Tris, pH 8.0, 5 mM Mg acetate and 1% protease inhibitor. Cells were dissolved in the buffer and vortexed for 5 min.

2. Sonication – cells were dissolved in the normal lysis buffer (as No. 1) and sonicated in a water bath for 5 min.

3. Urea only lysis buffer – 9 M urea, 4% CHAPS, 10 mM Tris, pH 8.0, 5 mM Mg acetate and 1% protease inhibitor. Cells were dissolved in the buffer and vortexed for 5 min.

Samples were centrifuged at 13000 rpm (Microcentaur, Sanyo) for 30 min at 4^0C to remove any insoluble materials e.g. cell debris. The protein concentration of the pooled supernatant of control and treated cells was determined using Bio-Rad Bradford assay (Section 2.4.2). This was then aliquoted accordingly and stored at -20^0C until use.

2.10.2 Two dimensional (2-D) gel electrophoresis

2-D gel electrophoresis was performed according to the manufacturer's instructions using standard Amersham Biosciences 2D-polyacrylamide gel electrophoresis (PAGE) apparatus and the PlusOne reagents (Buckinghamshire, UK). The total protein loading for analytical and preparative gels was 50 µg and 400 µg, respectively. Cell lysates were diluted with rehydration buffer (Appendix D.2) to a total volume of 350 µl and applied onto the Immobiline pH gradient (IPG) strips (pH 3-10 non-linear, 4-7 linear or 6-9 linear, 18 cm) using an in-gel rehydration technique (Sanchez *et al.*, 1997). This involved the reswelling of dry IPG strips by placing the gel side down directly into the rehydration solution containing the sample. This was subsequently overlayed with IPG cover fluid and in-gel rehydration was carried out overnight at RT.

2.10.2.1 Isoelectric focusing (IEF)

First dimensional electrophoresis, IEF was carried out on a Multiphor II electrophoresis unit consisting of stepped voltages from 500 to 3500 V. Different total focusing times were tested using a total volt-hours of 30000 (for IPG strips 6-9 only), 45000, 60000, or 100000 (Appendix D.3). After IEF, the IPG strips were stored at -20^0C until second dimension electrophoresis was performed.

2.10.2.2 IPG strip equilibration

Prior to 2-D gel electrophoresis the IPG strips were equilibrated. IPG strips were carefully placed in the rehydration tray with the gel side facing up and then incubated in SDS equilibration buffer 1 containing DTT (Appendix D.4, 2 ml per strip) for 15 min at RT with slow shaking (Orbital mixer, Denley). After aspiration, the above procedure was repeated using SDS equilibration buffer containing iodoacetamide instead of DTT (Appendix D.4).

2.10.2.3 Second dimensional gel electrophoresis

Sodium dodecyl sulphate (SDS)-PAGE was performed according to the method of Laemmli (1970) in a vertical gel electrophoresis tank (Hoefer SE600 apparatus). Homogeneous polyacrylamide gels containing 12% T acrylamide (Appendix D.5) were prepared using a multiple gel (18 X 16 X 0.15 cm) caster according to manufacturer's instructions.

Immediately after equilibration, the IPG strip was embedded on top of the SDS-PAGE gel, sealed with 2% molten agarose (Appendix D.6) and assembled into the electrophoresis tank filled with running buffer (Appendix D.7). The gel was run initially at 25 mA/gel until the blue dye entered the resolving gel and then the current was increased to 40 mA/gel. The gel electrophoresis run was terminated once the dye front migrated to the bottom of the gel. After second dimensional electrophoresis the gels were carefully transferred into a glass tray containing fixing solution and left O/N (Appendix D.8).

2.10.3 Protein staining

All analytical gels were silver-stained using PlusOne silver staining kit (Amersham Biosciences, UK) according to the manufacturer's instructions. Preparative gels were also silver-stained with the same kit using a modified protocol omitting the glutaraldehyde (Yan *et al.*, 2000). Gels were scanned immediately at 100 μm resolution using a personal SI Densitometer (Amersham Biosciences).

2.10.4 Gel analysis using computer-assisted 2-D software programme

The computer 2-D software package used was ImageMaster 2D Platinum software 5.0 (GE Healthcare/Amersham Biosciences). All procedures were essentially according to the manufacturer's user guide and detailed as follows:

1. Automatic spot detection with default parameters

2. Manual spot editing: add, erase, or combine spots if necessary to ensure the correct representation of the gel image. This was a time consuming step with possible human intervention.
3. Gel matching: a best resolved gel image was chosen as master. A number of landmarks were chosen for each gel. After automatic matching, extensive checking and editing were carried out.
4. Spot parameters: contained spot information such as spot number, pI and Mr. Spot quantity normalisation was performed using % spot intensity prior to statistical analysis.
5. Statistical analysis: gels were grouped as control and treated. Quantitative analysis was then performed for each group. Although the software carries out the t-test, it does not provide the probability (i.e. confidence interval).

2.10.5 Enzymatic digestion and mass spectrometry for protein identification

Spots were excised manually from each of two preparative gels. Excised spots were destained in 30 mM potassium ferricyanide and 100 mM sodium thiosulphate (1:1, freshly mixed) according to Yan *et al.*, 2000. Samples were washed alternatively with 50 mM ammonium bicarbonate, 50% acetonitrile in 50 mM ammonium bicarbonate and 100% acetonitrile. In-gel tryptic digestion was carried out at RT overnight using 0.25 µg of trypsin in 50 mM ammonium bicarbonate (pH 8.5).

Peptides were extracted sequentially using 1% TFA, 50% acetonitrile in 0.2% TFA and 100% acetonitrile. The pooled extraction solution was dried in a GyroVap centrifugal evaporator (Howe, Banbury, UK). For LC-MS/MS ion trap analysis (Vipond *et al.*, 2005), the dried digest was dissolved in 10 µl of solvent A containing 95% water, 5%

acetonitrile and 0.1% formic acid. The digest was separated in a 60 min LC run using solvent A and B (containing 95% acetonitrile, 5% water and 0.1% formic acid), on a HyPurity C18 column (100 x 0.18 mm, 3µ) (Thermo Electron, Hemel Hempstead, UK) with column flow rate of 2 µl/min. After initial isocratic flow of solvent A for 5 minutes, a gradient of 100% to 30% A/0% to 70% B was carried out for 36 min followed by a 4 min flow of 30% A/70% B, and 15 min of 100% A. Peptide sequencing was carried out using data dependent acquisition on a LCQ Deca XP Plus (Thermo Electron). The mass spectra were analysed and searched against the full entry of the UniProt/Swiss-Prot FASTA database, Release 49.0 (www.epi.ac.uk). The MS Spectra were also searched against the NCBI non-redundant protein database (May 2005, http://www.ncbi.nlm.nih.gov). Partial methionine oxidation was assumed and one enzyme miscleavage was allowed during searching. A protein was considered to be successfully identified where three or more peptide sequences from the MS data matched with a predicted protein from the two databases, and where at least one of the peptides passed the single threshold filter by Xcorr (1.50, 2.00, 2.50) vs charge state (+/-1,2,3) respectively. No taxonomic restriction was used for searching, and the species of origin for each protein was determined from detailed search results. Where multiple species were listed the protein was homologous between species of origin. Highest peptide coverage was usually observed for the protein of *Cricetulus griseus* (CHO) origin, when the sequence was available, and this accession number was reported. When matched peptides had 100% homology and protein isoforms could not be differentiated, the origin with higher species rank was reported (e.g reporting human rather than rat, rat rather than mouse, etc).

Chapter 3

Results

3.1 ADP-ribosylation assay: Standardisation and assessment of residual PT ADP-ribosylation activity in pertussis vaccines and its relationship with the *in vivo* HIST.

Cyr *et al* (2001) established an *in vitro* quantitative method using fluorescence detection of the tagged product for measuring the enzymatic activity of PT. Since the enzymatic activity of PT is considered essential for histamine sensitisation, the method was suggested as a potential alternative assay to the *in vivo* HIST. Subsequently, a direct correlation was seen between the ADP-ribosylation activities of native PT and its *in vivo* toxicity as measured by HIST (Yuen *et al*, 2002). Despite this, the relationship between the residual ADP-ribosylation activity of PT in pertussis or pertussis-containing vaccine formulations and their reactivity in the HIST has not been explored. This is essential in order to assess the possibility of replacing the HIST with the ADP-ribosylation assay. In the present study, the ADP-ribosylation assay has been further optimised, standardised and used to measure the activity in 51 batches of pertussis-containing vaccines of 10 different formulations. The enzymatic activities in these vaccines were compared with the results obtained from the HIST.

3.1.1 Selection of substrate for ADP-ribosylation assay

A fluorescently-labelled substrate for PT was purchased from two commercial sources. It differed from the original substrate as described by Cyr *et al.* (2000) by lacking the linker ($(CH_2)_5$) that joins the FAC to the 20 amino acid peptide (Chapter 2, Figure 2.1). The substrate without the linker (FAC-$G_{\alpha i3}$C20) was analysed and compared to the original substrate (FAC-$(CH_2)_5$-$G_{\alpha i3}$C20) to test its suitability as substrate for PT-catalysed ADP-ribosylation.

3.1.1.1 Characterisation of FAC-$(CH_2)_5$-$G_{\alpha i3}$C20 and FAC-$G_{\alpha i3}$C20 peptide substrates

The characterisation of FAC-$(CH_2)_5$-$G_{\alpha i3}$C20 peptide substrate and its ADP-ribosylated product using reverse-phase HPLC and mass spectrometry has been described previously (Cyr *et al.*, 2001; Yuen *et al.*, 2002). The FAC-$G_{\alpha i3}$C20 was reported by the manufacturer to be ≥ 90% pure. Further characterisation of FAC-$G_{\alpha i3}$C20 peptide substrate was done by mass spectrometry and chromatography. To investigate substrate isomers and impurities, a slower gradient-elution was used than that used for the ADP-ribosylation assay (Chapter 2, Section 2.7). Upon HPLC analysis, the FAC-$G_{\alpha i3}$C20 peptide substrate eluted as two peaks (Figure 3.1) and mass analysis showed that both compounds had the same mass and they are the 5/6-isomers of the fluorescein tag (results not shown). The other peaks are impurities from the substrate and, since they do not change during analysis of the ADP-ribosylated product peak, further investigation into the impurity peaks were not carried out. The two substrate isomers, however, were purified further by collecting the eluted peaks which were designated FAC-$G_{\alpha i3}$C20-1 and FAC-$G_{\alpha i3}$C20-2. PT-catalysed ADP-ribosylation of FAC-$G_{\alpha i3}$C20-1 and FAC-$G_{\alpha i3}$C20-2 was carried out and assayed using reverse-phase HPLC. The purified substrates, in terms of assay sensitivity, showed no differences in activities (results not shown). All further experiments were carried out using the mixture of FAC-$G_{\alpha i3}$C20-1 and FAC-$G_{\alpha i3}$C20-2 (FAC-$G_{\alpha i3}$C20).

A typical chromatogram showing the HPLC analysis of the ADP-ribosylated product is shown in Figure 3.2. The ADP-ribosylated product peak was well resolved from the substrate with retention times of 7.1 and 10.9 minutes respectively. This was similar to that reported by Yuen *et al* (2002). However, using FAC-$G_{\alpha i3}$C20, a co-elution impurity

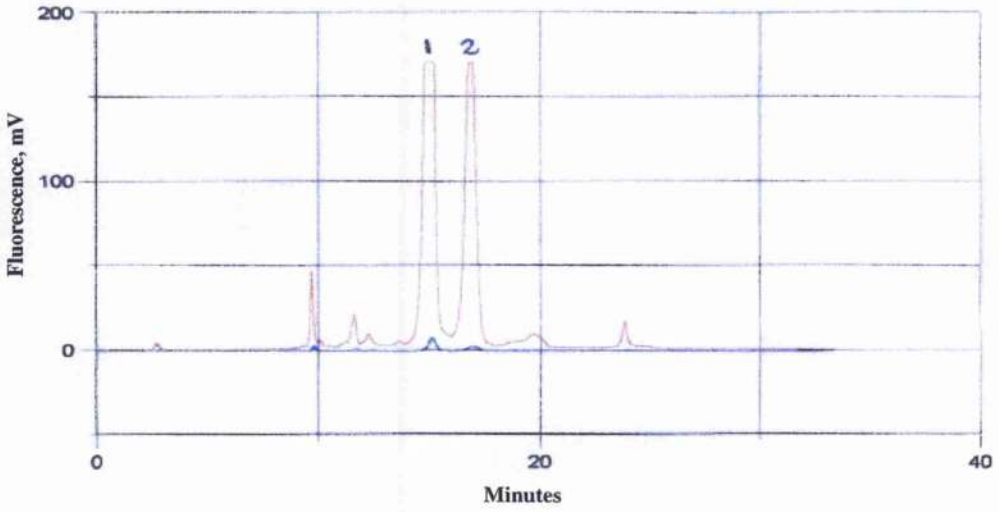


Figure 3.1. HPLC of FAC-$G_{\alpha i3}$C20 substrate. Fluorescence (red line) and UV (blue line). 1 µl of FAC-$G_{\alpha i3}$C20 peptide substrate solution (750 µM, as determined by UV absorption at 495 nm in 0.2 M Tris-HCl pH 7.6:DMSO (1:1)) was injected into the column and separation was achieved using a slow gradient-elution, as described in Chapter 2, Section 2.7.

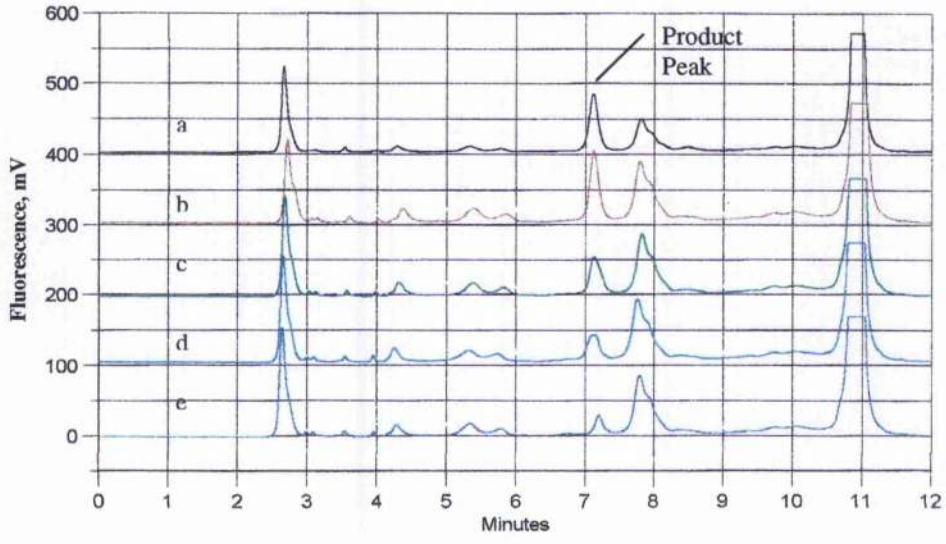


Figure 3.2. Typical chromatograms showing the product of the PT-catalysed ADP-ribosylation of FAC-$G_{\alpha i3}$C20 detected at 495 nm. Assay conditions were as described in the Section 2.7. The substrate peak was the "off scale" peak between 10.5 and 11.2 min. Trace (a) were obtained with 500 ng/ml PT 90/518 (5 µl injected to make sure it is within scale); Traces (b)-(e) were obtained with 250, 125, 62.5 and 0 ng/ml PT 90/518 respectively (10 µl injected).

substrate peak at 7.2 was observed in this study. It was not considered essential to improve the resolution between the product and impurity peak since this peak did not interfere with the final calculation of the enzymatic activities. The same consideration was applied to the other substrate impurity peaks at times: 4.2, 5.3, 5.7 and 7.8 min. The structures of the ADP-ribosylated product peak and FAC-$G_{\alpha i3}$C20 were confirmed by mass spectrometry and gave molecular masses ($M + H^+$) of 3153.3 (theoretical: 3155) and 2612.7 (theoretical: 2614) respectively.

3.1.1.2 Comparison of FAC-$G_{\alpha i3}$C20 and FAC-$(CH_2)_5$ - $G_{\alpha i3}$C20 as peptide substrates for PT

After ADP-ribosylation by PT, the FAC-$G_{\alpha i3}$C20 peptide without the linker showed a higher fluorescence reading hence a higher enzyme activity than the substrate with the linker and therefore a better sensitivity, by approximately 30% (average of two experiments) in comparison to FAC-$(CH_2)_5$-$G_{\alpha i3}$C20 (Figure 3.3). In addition, the relationship between the substrate concentration and ADP-ribosylation activities expressed in fluorescence units, using 250 ng/ml PT, showed good correlation with the correlation coefficient, r^2 being > 0.98 for both substrates. Hence, in the subsequent PT assays, the substrate without the linker was used.

3.1.2 Standardisation of ADP-ribosylation assay

Analytical characteristics of the ADP-ribosylation assay e.g. assay accuracy, sensitivity and precision for PT, have been described previously by Yuen *et al.*, (2002). In the present study, this assay system was further standardised for the purpose of determination of ADP-ribosylation activities in vaccine formulations. Two aluminium adjuvants are currently used in ACVs or ACV-based combination vaccines namely $Al(OH)_3$ and $AlPO_4$. The effect of these salts on the ADP-ribosylation activity of PT

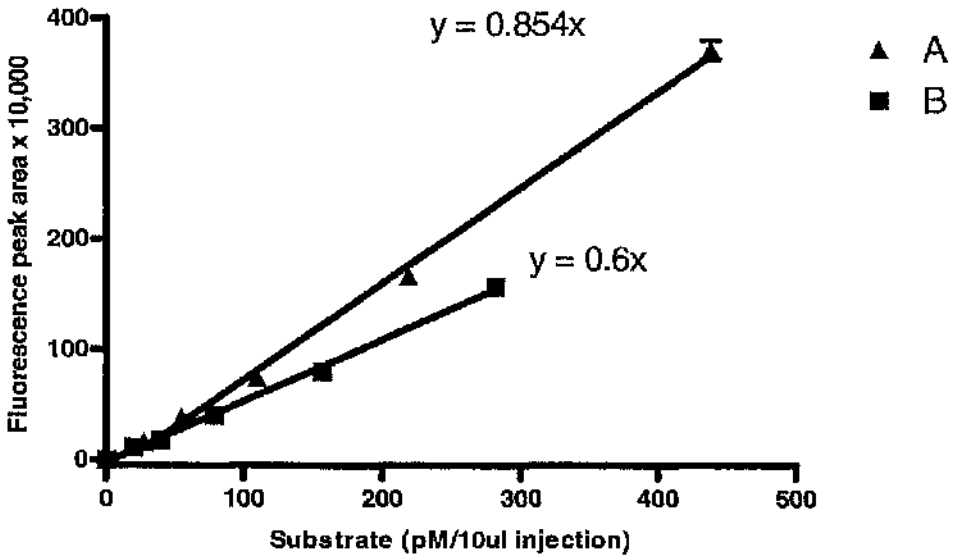


Figure 3.3. Comparison of FAC-$G_{\alpha i3}$C20 (A) and FAC-$(CH_2)_5$-$G_{\alpha i3}$C20 (B) peptides as substrates for PT (250 ng/ml)-catalysed ADP-ribosylation reaction. Results shown are the means of duplicate values.

was studied by adding equal volumes of PT 90/518 to the aluminium salts (0-13 mM). In the presence of $Al(OH)_3$ (5 mM), the ADP-ribosylation activity of 90/518 (500 ng/ml) decreased by approximately 50% with the inhibitory effect at a maximum at ≥ 5 mM. This effect was seen using either FAC-$G_{\alpha i3}$C20 or FAC-$(CH_2)_5$-$G_{\alpha i3}$C20. In contrast, no reduction in the ADP-ribosylation activity was seen with $AlPO_4$ (results not shown). The dose-response curves for PT (0-1000 ng/ml) with these two adjuvants (13 mM) were assessed in four independent assays. Although, with both types of adjuvants, there was high linearity ($r^2 > 0.98$) (Figure 3.4), the slopes for the two types of standard-curves were significantly different ($p < 0.05$) and a higher slope value (mean = 0.22) was found with $AlPO_4$ than with $Al(OH)_3$ (mean = 0.15). Therefore, in each ADP-ribosylation assay, an appropriate PT standard curve was constructed using either $Al(OH)_3$ or $AlPO_4$ adjuvant as diluents for PT 90/518, according to the type of adjuvant present in the test sample.

The reason for the apparent change in enzymatic activities in the presence of the different aluminium salt solutions was not clear. The effect due to pH difference was excluded since the pH was found to be similar in the two aluminium salt solutions. The loss of fluorescence due to adsorption to $Al(OH)_3$ was also investigated as, prior to the separation of the ADP-ribosylated product by reverse-phase HPLC, the enzymatic reaction mixture was centrifuged to remove aluminium salt entering the column that could block the column flow. Freshly prepared $Al(OH)_3$ was added to a sample of ADP-ribosylated product of known enzyme activity and analysed by HPLC in the same way as before. No difference in the level of fluorescence was observed after the inclusion of $Al(OH)_3$ (results not shown).

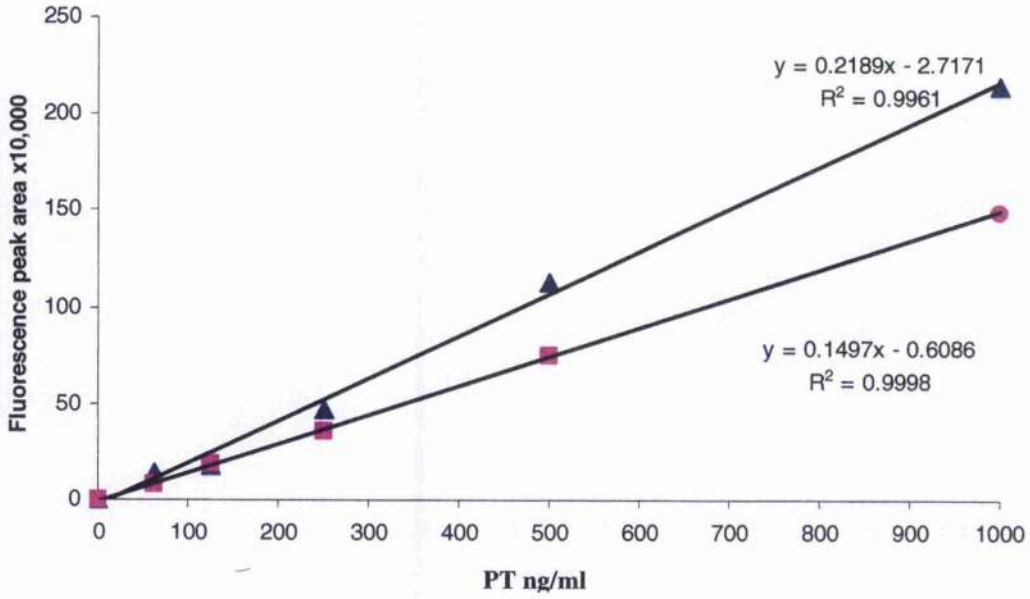


Figure 3.4. Comparison of dose-response curves for ADP-ribosylation by PT in the presence of adjuvants: ▲ $AlPO_4$; ■ $Al(OH_3)$; ● extrapolated value.

The vaccine preparation, Biken (Section 2.7.1) containing two pertussis components (PT, FHA) in combination with D and T was used as the positive control vaccine in each ADP-ribosylation assay. The repeatability of the assays was assessed by 4-6 independent assays using the ratio of ADP-ribosylation activities of the PT reference at concentrations of either 250 or 125 ng/ml compared to the ADP-ribosylation activities in 1 SHD of the positive control vaccine. The analytical characteristics of the assay method are presented in Table 3.1 and validity criteria for the ADP-ribosylation assay have been set as follows: the negative control (0 ng PT) should show no ADP-ribosylation activity; the standard curve of PT constructed with either $Al(OH)_3$ or $AlPO_4$ should show linearity (r^2>0.96); the ratio of PT at 250 ng/ml reconstituted with $Al(OH)_3$ or $AlPO_4$ adjuvant to the positive control vaccine preparation (00/486) should be 0.018 (0.016-0.02) and 0.025 (0.015-0.035) at 95% confidence level, respectively. The data presented in this study were all obtained from assays that met these validity criteria, unless indicated.

The specificity of the ADP-ribosylation assay was examined by assessing the enzymatic activity in other vaccine components which are commonly present in ACV-based combination vaccines, e.g. IPV, PRP-T and also in a combination vaccine product DTaP-I, in which the ADP-ribosylation activity of PT had been eliminated by mutation. The specificity of the assay system for ADP-ribosylation activity due to PT was evidenced by the absence of enzymatic activity seen with DTaP-I or DTaP-I in combination with any of these other vaccine components (results not shown).

Table 3.1. Assay characteristics for ADP-ribosylation assay

Assay condition	*n*	Regression line				Ratio of fluorescence units with PT to that with the positive control vaccine					
		Linearity		Slope		Within –day			Day-to-day		
		Mean r^2	S.D.	Mean	S.D.	Mean	S.D.	CV%	Mean	S.D.	CV%
PT-$AlPO_4$ 0-1000ng	4	0.98	0.010	0.22	0.06	n/a	n/a	n/a	n/a	n/a	n/a
PT-$Al(OH)_3$ 0-500ng	4	0.98	0.008	0.15	0.004	n/a	n/a	n/a	n/a	n/a	n/a
PT-saline 125ng	6					0.021	0.002	9.8	nd	nd	nd
PT-saline 250ng	6					0.030	0.003	8.4	nd	nd	nd
PT-$AlPO_4$ 125ng	6					nd	nd	nd	0.012	0.003	26
PT-$AlPO_4$ 250ng	6					nd	nd	nd	0.025	0.005	21
PT-$Al(OH)_3$ 125ng	4					nd	nd	nd	0.008	0.001	13
PT- $Al(OH)_3$ 250ng	4					nd	nd	nd	0.018	0.001	8

PT: pertussis toxin 90/518; PT-$AlPO_4$ and PT-$Al(OH)_3$, pertussis toxin in $AlPO_4$ or $Al(OH)_3$ respectively. S.D.: standard deviation; CV %: coefficient of variation; *n*: number of assays. Data are expressed as the ratio of fluorescence units /PT (125 or 250 ng/ml) to fluorescence units / 1 SHD of control vaccine 00/486 under the experimental condition described in Chapter 2, Materials and Methods Section 2.7.3. n/a: not applicable, nd: not determined

3.1.3 Residual PT ADP-ribosylation activities in different pertussis vaccine formulations

Residual PT ADP-ribosylation activities were measured in 51 batches of vaccines in 10 different formulations and the results are presented in Table 3.2. ADP-ribosylation activities were detected in all vaccine formulations except DTaP-I. However, no consensus average enzyme activity could be defined amongst these different vaccine formulations and the range of activity was wide between the products, ranging from 0 (DTaP-I) to 10.2 (in one batch of DTaP-D) E-units/SHD. Nevertheless the level of residual PT enzyme activity in DTaP vaccine formulations appeared to be product-specific and batch-to-batch variability (CV %) ranged from 22.5 to 34 % (Figure 3.5) where the number of batches tested for a particular product was ≥ 6. Similar to previous findings (Cyr *et al.*, 2001), the residual PT enzymatic activity measurable in different batches of WCV (DTPw-J) was found to be much lower in comparison to DTaP, with the exception of DTaP-G which is a booster vaccine that contains a much lower amount of PT antigen in comparison to other DTaP formulations (Chapter 2, Table 2.1).

3.1.4 Comparison of residual PT ADP-ribosylation activities and their relationship with the *in vivo* HIST

All the above DTaP vaccine batches were also tested by the HIST and a comparison of their PT ADP-ribosylation activities and their reactivity in HIST is shown in Figure 3.5. As expected, two batches of DTaP-I product, which did not have detectable enzymatic activity, did not show any reactivity in HIST. Products DTaP-G and DTaP-H which had low or

Table 3.2 ADP-ribosylation activities in different pertussis vaccine formulations.

	ADP-ribosylation activity units per SHD*									
Product code	**DTaP-A**	**DTaP-B**	**DTaP-C**	**DTaP-D**	**DTaP-E**	**DTaP-F**	**DTaP-G**	**DTaP-H**	**DTaP-I**	**DTPw-J**
Number of batches tested	9	1	6	14	3	3	2	1	2	10
Mean	1.51	1.67	3.28	4.66	6.58	6.50	0.10	0.46	0	0.18
Range	0.91-2.58	n/a	2.41-4.75	2.86-10.2	6.41-6.78	5.46-7.35	0.10	n/a	n/a	0.12-0.32

*ADP-ribosylation activity unit per SHD of test vaccine, where one E-unit is equivalent to the enzymatic activity produced by 1 μg of PT. n/a, not applicable.

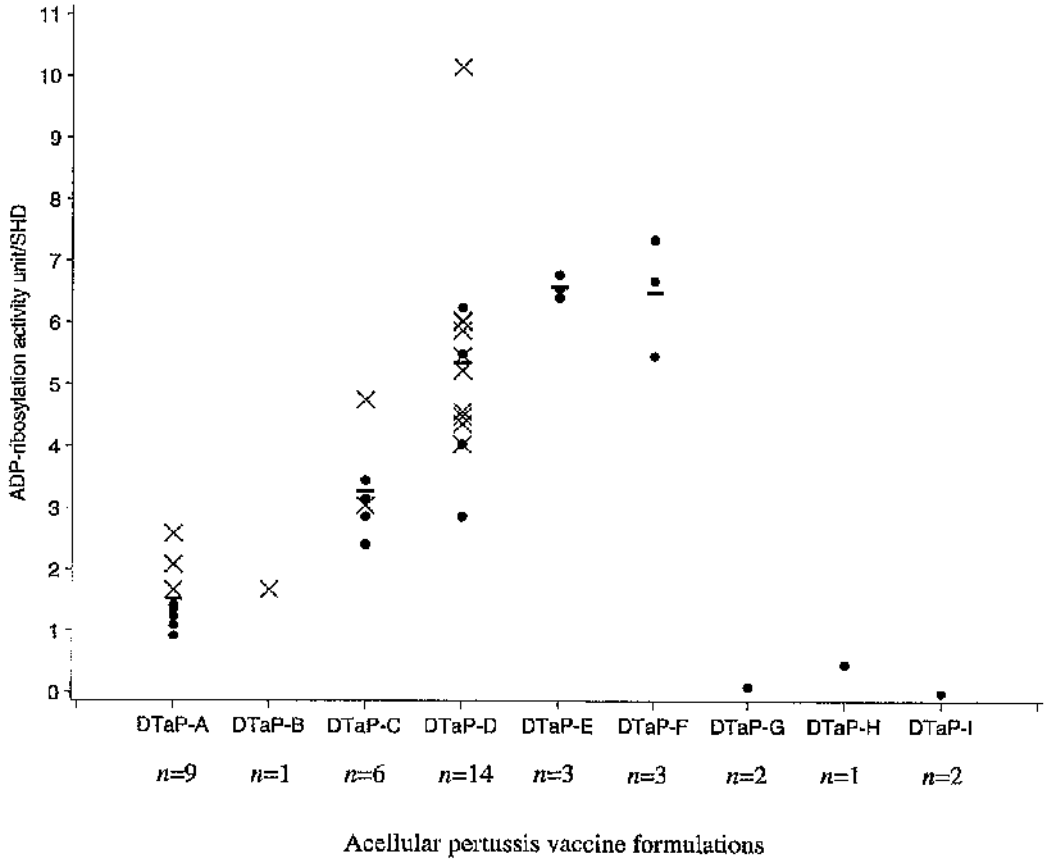


Figure 3.5. ADP-ribosylation activities in different pertussis vaccine formulations and relationship to activities in the in vivo HIST. The average value per group of vaccines is represented by a '-' and crosses indicate vaccine batches that show reactivity in the HIST and dots indicate batches that were non-reactive in HIST. The results were expressed as ADP-ribosylation activity units (E-units) per SHD of test vaccine where 1 E-unit is equivalent to the enzymatic activity produced by 1 µg of PT (90/518)

modest levels of ADP-ribosylation activities, did not show reactivity *in vivo* although only 1 or 2 batches of these vaccines were tested. A positive relationship between the enzyme activity in vaccine lots and the *in vivo* reactivity observed by the HIST was found for product DTaP-A where 3 lots had higher enzymatic activities and also showed reactivity in mice. However, this was not the case for products DTaP-C to DTaP-F where no positive relationship between the *in vitro* and *in vivo* systems could be found, either between these vaccine formulations or between different lots within the same type of product. The most striking finding was with products DTaP-E and DTaP-F. These showed the highest mean enzymatic activities but no batches showed any reactivity in the *in vivo* HIST. This can be compared with product DTaP-D which had similar or lower enzyme activities, but the majority of batches showed sensitising reactivity in HIST. The results indicate that a value of enzymatic activity common to all vaccines and predictive of *in vivo* reaction could not be defined.

Interestingly, product DTaP-C was an intermediate bulk for the DTaP-D vaccines, but the latter product had been formulated with other additional antigen components. A comparison was made between these two products using 4 paired batches from each vaccine. Batches of product DTaP-D that sensitised mice in the HIST, displayed higher enzyme activities by approximately 30% than the corresponding DTaP-C batches that showed no reactivity *in vivo* (Figure 3.6). The observed difference in enzymatic activities was not attributed to other antigen components since the assay system was specific for PT in that no enzymatic activity was seen with any of the other components tested. Moreover, addition of these antigen components to DTaP-C products did not enhance the enzymatic activities (results not shown).

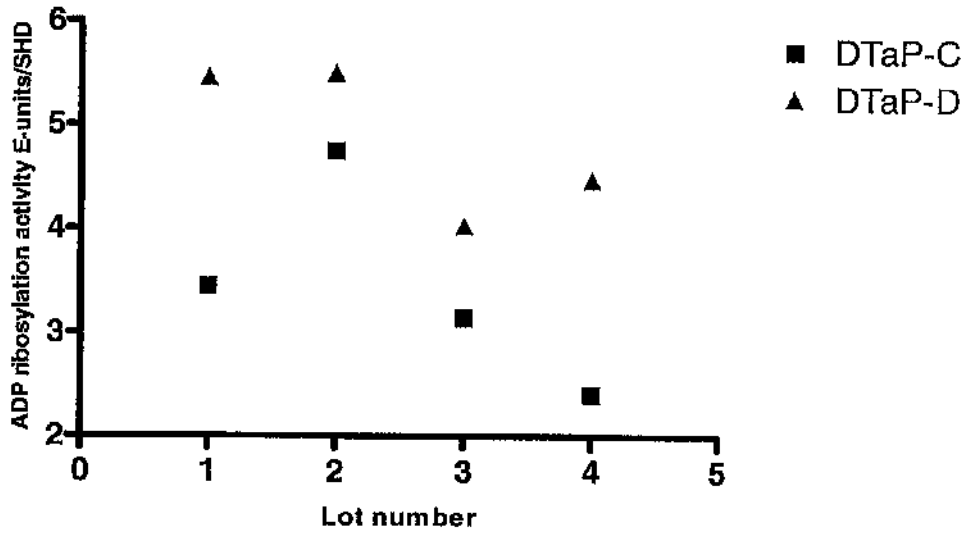


Figure 3.6. Comparison of ADP-ribosylation activities of paired DTaP-C and DTaP-D products. The results were expressed as ADP-ribosylation activity units (E-units) per SHD of test vaccine where 1 E-unit is equivalent to the enzymatic activity produced by 1 μg of PT (90/518).

3.2 Development of a carbohydrate-binding assay for pertussis toxin and toxoid

The ADP-ribosylation assay described in the previous section measures only the A-subunit activity of PT. The precise mechanisms and sites of different detoxification procedures on the PT molecule are not known, thus the PT/d present in vaccines could be modified at different sites in the B-oligomer, A-subunit, or both. Since both A- and B-subunits contribute to histamine sensitisation and both of these functions are assessed in the *in vivo* toxicity control test for assessing residual PT activity in vaccines, the use of the ADP-ribosylation assay alone, therefore, may not reflect the activity of PT observed in HIST. In the following section, a carbohydrate-binding assay was developed for measuring the B-subunit activity of PT.

3.2.1 Characterisation of biotinylated (BTN-) oligosaccharides

Since native oligosaccharides do not adhere directly to microwells, oligosaccharide structures were derivatized with an amino-biotin derivative (Chapter 2, Section 2.8.4). Typical HPLC chromatograms of the BTN-oligosaccharides are shown in Figure 3.7 and demonstrate high efficiency of the biotinylation reaction and good separation of the biotinylated products from the starting materials. NA3-BTN gave a single major peak at 17.5 min, which showed >95% completion of the biotinylation reaction and NA3 oligosaccharide eluted at 21 min as a very minor peak. The HPLC chromatogram of A3-BTN showed a major product eluted at 32.5 min and two smaller peaks at 27.5 and 30.3 min. Mass analysis indicated the latter were BTN-oligosaccharides generated from other minor *N*-glycans present in the starting material (data not shown). Mass analyses of all the BTN-oligosaccharides used in the binding assays are presented in Table 3.3.

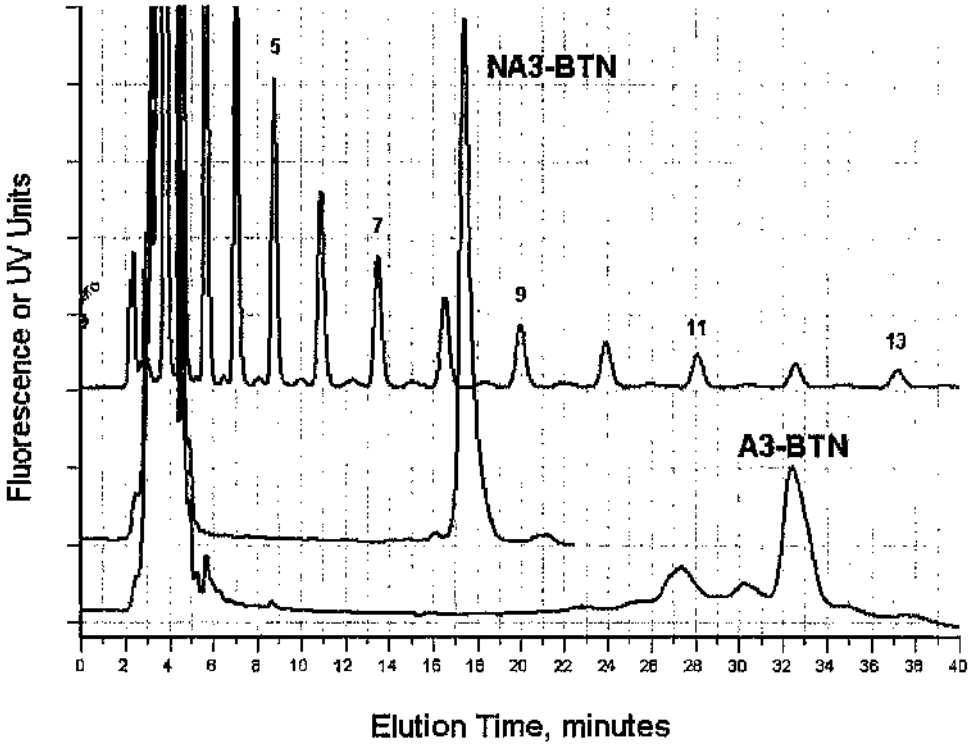


Figure 3.7 Typical Amide-80 HPLC chromatograms of A3-BTN and NA3-BTN after partial purification by ethyl acetate extraction. The detection of the BTN-oligosaccharides was by UV at 223nm and the detection of the glucose oligomer ladder of 4-aminobenzoic acid derivatives (in numbers; Yuen *et al.*, 2002) was by fluorescence ($\lambda_{Excitation}$, 296nm; $\lambda_{Emission}$, 359nm).

Table 3.3. Monosaccharide compositions, approximate hexose contents and mass spectrometry data of BTN-glycan structures.

BTN-glycans	Structures	Monosaccharide composition	Hexose Approx. %	Calculated mass $[M+H]^+/[M+Na]^+$	Found mass $[M+H]^+/[M+Na]^+$
NA2	Asialo biantennary	$Gal_2Man_3GN_4$	42.1	1925.90/1947.90	1925.81/1947.86
NA3	Asialo triantennary	$Gal_3Man_3GN_5$	42.5	2291.24/2313.24	2291.26/2313.25
NA4	Asialo tetraantennary	$Gal_4Man_3GN_6$	42.7	2656.58/2678.58	2656.58/2677.82
A1	Mono-sialylated biantennary	$SaGal_2Man_3GN_4$	36.6	2217.16/2239.16	2216.99/2238.50
A2	Di-sialylated biantennary	$Sa_2Gal_2Man_3GN_4$	32.3	2508.42/2530.42	2508.47/2530.48
*A2-1	Di-sialylated (1Ngc) biantennary	$Sa_2(1Ngc)Gal_2Man_3GN_4$	32.1	2524.42/2546.42	2524.89/nf
*A2-2	Di-sialylated (2Ngc) biantennary	$Sa_2(2Ngc)Gal_2Man_3GN_4$	31.9	2540.42/2562.42	2540.73/nf
*A2-3	Tri-sialylated (2Ngc) biantennary	$Sa_3(2Ngc)Gal_2Man_3GN_4$	28.8	2831.68/2853.68	2832.07/nf
*A2-4	Tri-sialylated (3Ngc) biantennary	$Sa_3(3Ngc)Gal_2Man_3GN_4$	28.5	2847.67/2869.68	2848.25/nf
A3	Tri-sialylated triantennary	$Sa_3Gal_3Man_3GN_5$	30.7	3165.02/3187.02	3165.23/3187.37
*A3-1$^+$	Tri-sialylated (1Ngc) triantennary	$Sa_3(1Ngc)Gal_3Man_3GN_5$	30.6	3181.02/3203.02	3181.99/nf
A3F	Tri-sialylated triantennary +core fucose	$Sa_3Gal_3Man_3GN_5F$	28.9	3311.16/3333.16	3308.0/nf
A4	Tetra-sialylated tetraantennary	$Sa_4Gal_4Man_3GN_6$	29.7	3821.62/3843.62	3824.0/nf
A4F	Tetra-sialylated tetraantennary +core fucose	$Sa_4Gal_4Man_3GN_6F$	32.3	3967.76/3989.76	3964.0/nf

Table 3.3. continued

BTN-glycans	Structures	Monosaccharide composition	Hexose Approx. %	Calculated mass $[M+H]^+/[M+Na]^+$	Found mass $[M+H]^+/[M+Na]^+$
Glc5	Maltopentaose	Glc_5	72.8	1113.1/1135.1	1113.6/1135.6
Lac	Lactose	GalGlc	51.8	626.68/648.68	627.69/649.67
SaLac	Sialyllactose	SaGalGlc	35.3	917.94/939.94	918.72/940.79

Glycoproteins	Major glycan structures found		Monosaccharides composition	Hexose Approx. %
**Fetuin	Multi-sialylated triantennary	A3, Sa_2NA3	$Sa_{(2-3)}Gal_3Man_3GN_5$	33
**RNase B	Oligomannose 5-8	Man(5-8)	$Man_{(5-8)}GN_2$	55

Abbreviations: F, fucose; Gal, galactose; Glc, glucose; GN, N-acetylglucosamine; Man, mannose; Ngc, N-glycolyl; Sa, sialic acid (N-acetylneuraminic acid); nf, not found. *structures are as defined in Materials and Methods section. ** Mixture of N-glycans released from glycoproteins [+]This fraction also contained $Sa_3(2Ngc)Gal_3Man_5GN$

3.2.2 Screening of glycoproteins and oligosaccharides as ligands for PT binding using ELISA

Prior to carrying out the oligosaccharide and glycoprotein binding experiments, non-specific binding of other components or reagents used in the system i.e. Neutravidin, biotin, primary and secondary antibodies was assessed. No detectable binding activity for these reagents was observed under current experimental conditions (data not shown). A panel of glycoproteins (Chapter 2, Section 2.8.2) with different oligosaccharide structures was investigated to test their suitability as coating ligands for PT using ELISA. The PT binding to these glycoproteins was found to be in a glycoprotein concentration-dependent manner. However, the binding of PT to the glycoproteins were also found to be structure dependent e.g. the binding of PT (25 ng/well) to hAGP, BTG, hTf, bAGP and Hg did not reach maximum even at a coating concentration of 8 μg/well whereas binding to Fet was maximum above coating concentration of 1 μg/well (Figure 3.8). When all the glycoproteins were used at 1 μg/well for coating, PT showed highest affinity to Fet whereas it bound moderately (relative to Fet) to hHg (48%), bTG (39%) and AFet (28%) (Figure 3.9a). Interestingly under these conditions, little or no binding was observed to glycoproteins hTf and bAGP, comprising mainly sialylated biantennary *N*-glycans. As expected, PT did not bind to RNaseB, that contains only neutral *N*-linked oligomannose 5-9 structures, nor to the non-glycosylated RNaseA or BSA (Figure 3.9 a).

A panel of oligosaccharides structures varying from neutral to acidic milk oligosaccharides and *N*-glycans was screened (Table 3.3, Figure 3.9b). PT had a strong preference for sialylated multi-antennary *N*-glycan structures (A3 and A4) and desialylation caused reduction in % binding, relative to that of A3, as in asialo tri-

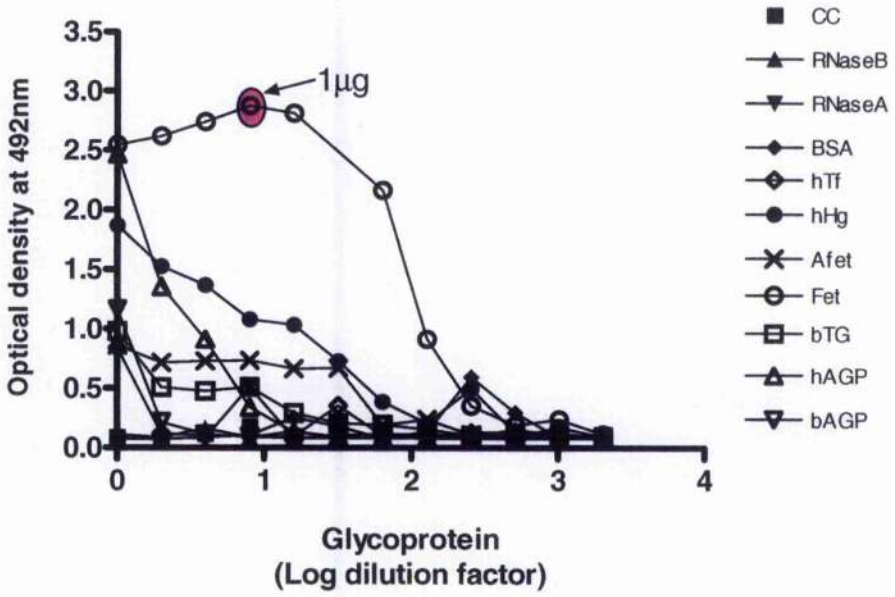
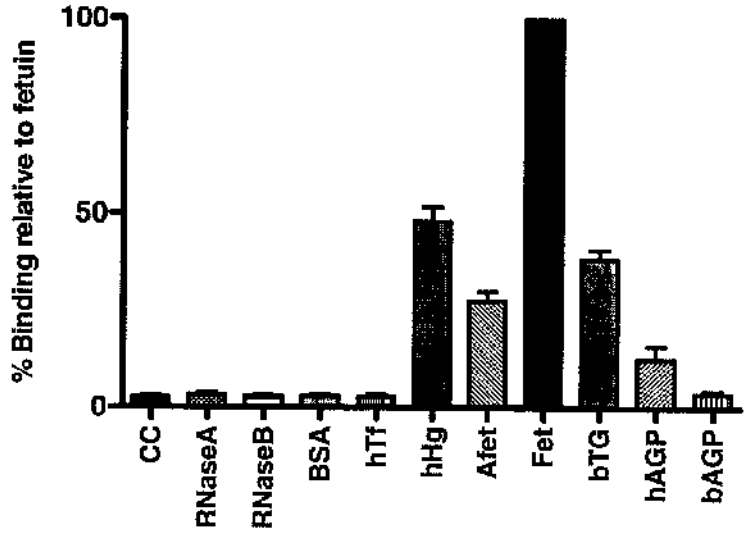


Figure 3.8. Binding of PT to glycoproteins coated at a starting concentration of 80 µg/ml (8 µg/well). Two-fold serial dilutions were carried out. PBST containing 3% BSA was used instead of PT for control wells coated with carbonate coating buffer (CC)

(a)



(b)

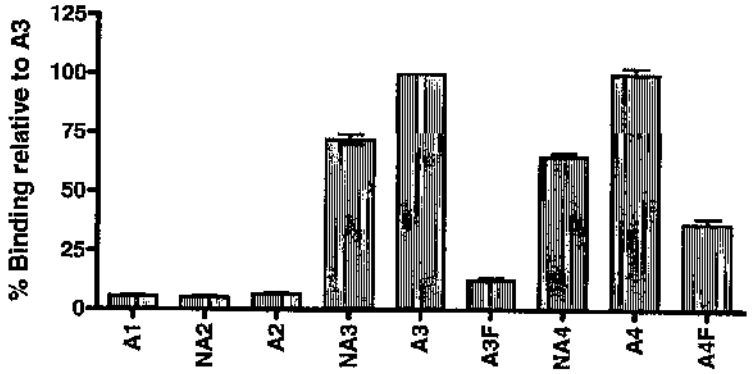


Figure 3.9. (a) Binding of PT to glycoproteins (as defined in Chapter 2, Section 2.8.2) Wells were coated with glycoproteins (1 µg per well in PBST containing 3% BSA) and binding of PT was detected as described Chapter 2, Section 2.8.7. CC, carbonate coating buffer. Results shown are the average of two experiments. For comparative purposes binding of PT to Fet was taken as 100 %. (b) Binding of PT to subgroups of BTN-*N*-glycans (as defined in Chapter 2, Section 2.8.3). Binding of PT was detected as described in Chapter 2, Section 2.8.8. Results shown are the means and standard error of means of three experiments. For comparative purposes binding of PT to A3 was taken as 100 %.

(NA3, 72%), tetra- (NA4, 65%) antennary structures. Moreover little or no binding was seen with mono- and di-sialylated or asialo- biantennary structures, which supported the results found in the glycoprotein binding experiment where little binding was observed with glycoproteins containing mainly biantennary *N*-glycan structures (hTf and bAGP; Chapter 2 Table 2.3). The addition of core fucose to both A3 and A4 structures reduced the % binding, relative to A3, to 13% (A3F) and 37% (A4F) respectively (Table 3.3, Figure. 3.9b). Milk oligosaccharides SaLac and Lac showed little binding to PT and neutral high-mannose structures (RNaseB) and maltopentaose (Glc5) showed only background binding. Binding of toxin also appeared to be greatly influenced by the type of sialic acid present since binding to A3 containing the *N*-glycolyl form of sialic acid (A3-1, Table 3.3) was 70% lower in comparison to A3 containing only the *N*-acetyl form of sialic acid (results not shown). In theory, A4 could be used but it is more expensive commercially. All further experiments therefore were carried out using oligosaccharides A3 and NA3.

3.2.3 Screening of glycolipids as ligands for PT

Previous studies have documented the binding of PT to gangliosides prepared from macrophages and cilia using TLC (Saukkonen *et al.*, 1992). Based on these observations, different glycolipids or neoglycolipids (Chapter 2, Table 2.2) with differing compositions were tested for their ability to bind PT. No binding was observed with any of the glycolipids tested (GM1, AGM1, GM2, GM3, data not shown) under the same experimental conditions for the glycoprotein-capture ELISA. Further investigations using the neoglycolipid capture-ELISA (Chapter 2, Section 2.8.6) ± Tween-20 from reagents to improve coating efficiencies did not result in observable binding to any of the glycolipids and neoglycolipids studied (results not shown). On the

contrary, using dot blotting, only very weak binding was observed to glycolipids GT1b and GD1a (Figure 3.10). Since the binding was weak, no further investigation for the purpose of developing the binding assay was carried out.

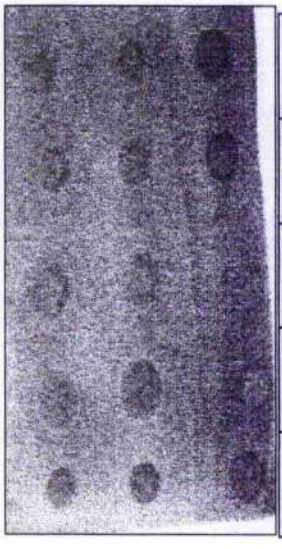
3.2.4 Comparison of PT and PTd binding in glycoconjugate-capture ELISA

The binding of PT to Fet-coated plates showed good dose-responses with a linear range from 0.5ng-30 ng/well. In contrast, the binding activity of a PTd detoxified by glutaraldehyde was much lower. With PTd at 1,000 ng/well, binding activity was equivalent only to that of 1.2 ng PT i.e. 850 times lower than PT (results not shown). When binding activities of PT and PTd were investigated using defined *N*-glycans, major differences in binding specificity of PT and PTd were observed (Figure 3.11). The binding activity of PTd to A3 was almost undetectable (0.4%) and at least 250 times less than that of PT at the same protein concentration (1.25 μg/ml). However, when NA3 was used to coat the ELISA plates, the binding activity of PTd was only 3 times lower than that of PT (Figure 3.11).

3.2.5 Effect of other vaccine components on the binding assay

Apart from PT/PTd, a variety of antigens such as FHA, PRN, Fims2/3, T, D, IPV and PRP-T can be present in different types of acellular pertussis combination vaccines. It was important therefore to investigate possible interference of these antigens in the PT binding assay. To address this issue, two approaches were undertaken in the present study: 1) The binding activities of these antigens to Fet were determined by using antibodies specific for these components; (2) the possible competition between these antigens and PT for the Fet binding sites was investigated. Apart from PT, none of the

GM1	GM2	GM2
GT1b	GT1b	GM1
GA1	GM3	Glu-cer
GA1	GM3	Glu-cer
DHPE	Sa3LeA5	Su3LeA5



GM2	GM2	GT1b
Glu-cer	Glu-cer	GT1b
DHPE	DHPE	GD1b
GM3	GM3	GD1b
GD1a	GD1a	GD1a

Figure 3.10. Binding of PT to (neo)glycolipids. 1 μg of indicated glycolipids was applied directly to nitrocellulose. Dot blots were then incubated with 1 μg/ml PT 90/518 and binding was visualised as described in Chapter 2, Section 2.8.5. Structures of glycolipids are shown in Chapter 2, Table 2.2

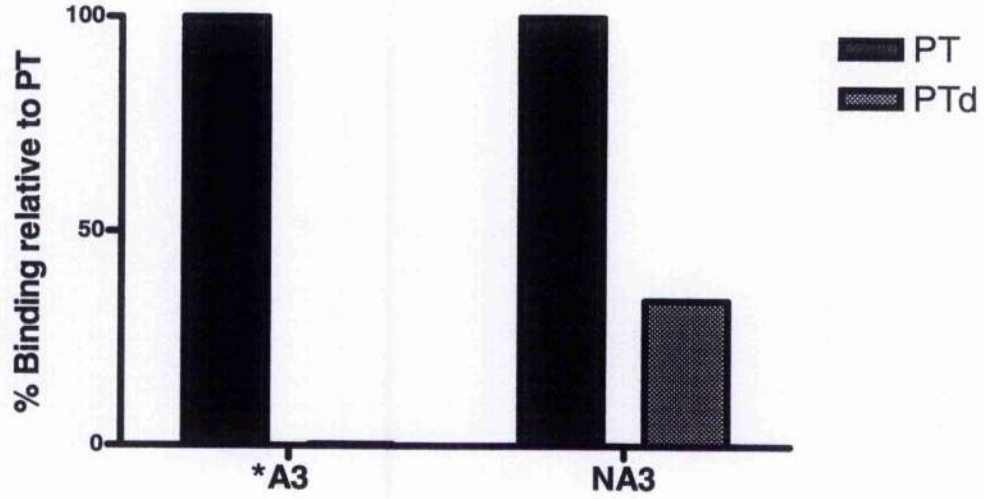


Figure 3.11. Binding of PT and PTd to oligosaccharides A3 and NA3 using oligosaccharide-ELISA. Binding activities were analysed by parallel line assay as described in Chapter 2, Section 2.8.9. For comparative purposes, binding of PT was taken as 100%. Results shown are the average of duplicate or *triplicate dose response curves.

antigens bound to Fet (results not shown). Moreover, addition of these antigens to PT either individually or in combination in a relative concentration typically present in vaccines, did not affect its binding to Fet (Figure 3.12).

3.2.6 Desorption of antigens from aluminium adjuvant solution

In pertussis-based combination vaccines, PT/d is adsorbed in varying degrees onto $Al(OH)_3$ or $AlPO_4$ adjuvant. Since these adjuvants interfere with the ELISA assay system (Doel and Staple, 1982; Katz, 1987), PT/d is required to be released (desorbed) from the aluminium adjuvant prior to the binding assay. Three desorption reagents: citrate, EDTA and Tween-20/EDTA were investigated for their ability to elute the antigen and PBS was used as a control. These desorption reagents had no effects on the PT binding assay system (results not shown). Since it was found that PT was more strongly adsorbed to $Al(OH)_3$ (>95%) than to $AlPO_4$ (<25% data not shown), it was essential to select an eluting reagent that was most effective for eluting antigen from the $Al(OH)_3$ adsorbed vaccines. Tween-20/EDTA was found to be unsuitable because, although it showed similar eluting efficiency in comparison to EDTA for $AlPO_4$-based vaccines, it was less effective for $Al(OH)_3$-based vaccines (results not shown). Furthermore, unexpectedly high ADP-ribosylation activities were observed after treatment of DTaP products with Tween-20/EDTA while citrate or EDTA alone showed little or no effect (Figure 3.13). Although EDTA gave slightly better results than citrate for $AlPO_4$-adsorbed vaccines, citrate was selected to be the eluting reagent because it was nearly 3 times more effective than EDTA in eluting PTd from $Al(OH)_3$ (Figure 3.14).

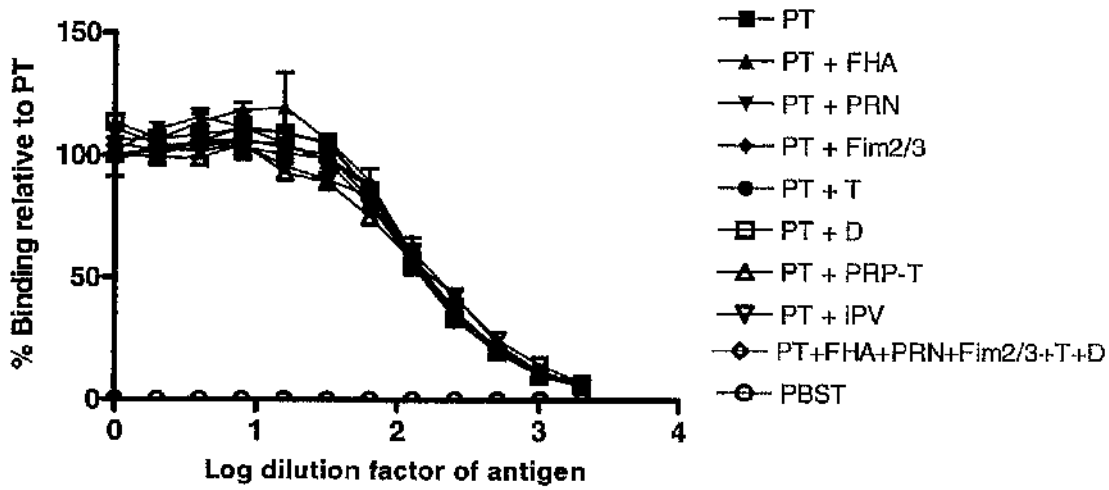


Figure 3.12. Binding of PT to Fet in the presence of other vaccine components. Wells were coated with 1 µg Fet, blocked and incubated with 400 ng/well PT, 400 ng/well FHA, 128 ng/well PRN, 100 ng/well Fims2/3, 100 µl of 4Lf T, 20 Lf D, 1/5 of IPV and 2.5 µg PRP-T. This was followed by incubation with anti-PT antibodies and was developed as described in Chapter 2, Section 2.8.7. Results shown are the average of two experiments. Lf, Limit flocculation unit.

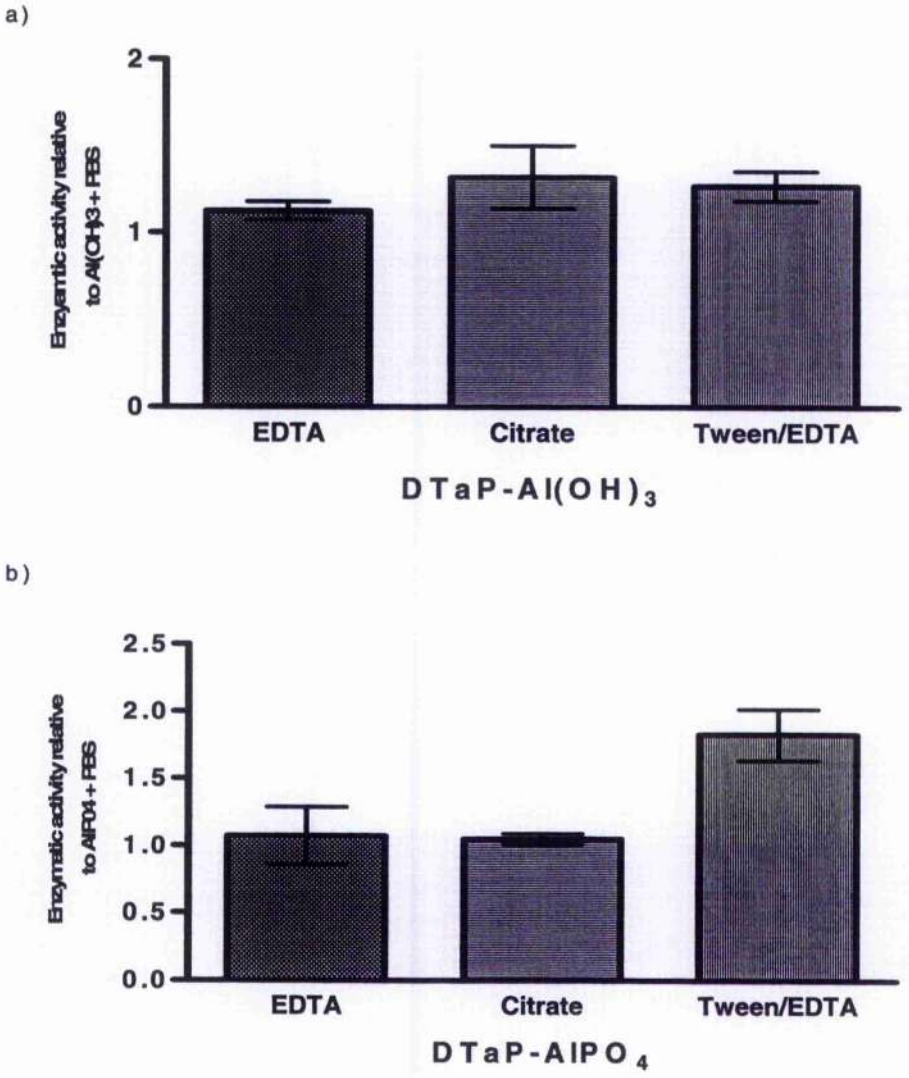


Figure 3.13. Effect of desorption reagents on ADP-ribosylation activity in DTaP preparations. For controls, DTaP-$AlPO_4$ (DTaP-C) or DTaP-$AlOH_3$ (DTaP-A) was treated with PBS, and the enzymatic activity was taken as 1. The enzymatic activities of DTaP-$AlPO_4$ and DTaP-$AlOH_3$ products after treatment were compared to those from DTaP-$AlPO_4$ and DTaP-$AlOH_3$ treated with PBS, respectively. Data represent the mean of three different batches of DTaP-$AlPO_4$ and three different batches of DTaP-$(AlOH)_3$ products, testing each condition in triplicate.

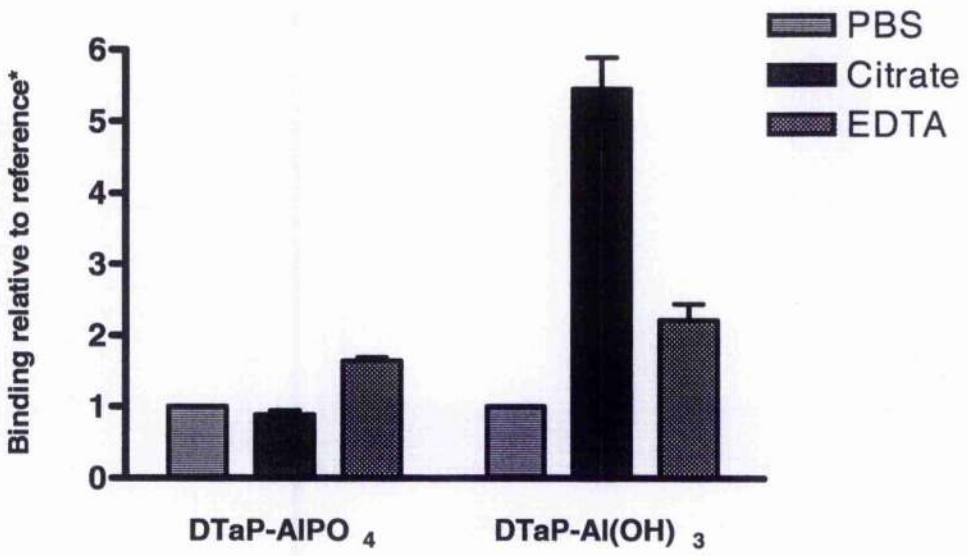


Figure 3.14 Fetuin binding activity of PTd eluted from $Al(OH)_3$ and $AlPO_4$ adsorbed vaccine preparations (DTaP, acellular pertussis vaccine preparation) using different desorption agents. *As controls, DTaP-$AlPO_4$ (DTaP-C) or DTaP-$Al(OH)_3$ (DTaP-A) was treated with PBS, and the binding activity of the PTd in the resulting supernatant fraction was taken as 1. The binding activities of DTaP-$AlPO_4$ and DTaP-$Al(OH)_3$ products after treatment were compared to those from DTaP-$AlPO_4$ and DTaP-$Al(OH)_3$ treated with PBS, respectively. Data represent the mean of three different batches of DTaP-$AlPO_4$ and three different batches of DTaP-$(AlOH)_3$ products, testing each condition in triplicate.

3.2.7 Validation of carbohydrate-binding assay using A3-capture ELISA

Since Fet, A3 and NA3 were found to be suitable ligands for PT binding in the ELISA system, validation of the binding assay was carried out using A3 coating as an example. The cut-off value was defined as the mean OD of the negative control plus 2 standard deviations (SD). The detection limit for PT binding under the experimental conditions was found to be 2 ng/well (n=11). Reference PT concentrations from 2.5 μg/ml to 1.2 ng/ml (500-0.24 ng/well) were examined and PT from 4-250 ng/well resulted in a linear dose-response curve ($r^2 = 0.99$). The day-to-day coefficients of variation of the ratio of the reference toxin (250-31.25 ng/well) to the blank were from 8.62 to 26.4 by 15 independent assays and the coefficient of variance increased as the PT concentration decreased. The intra-assay variation for PT was determined by comparison of 5 replications of dose-response curves within the same day. The potencies of the 5 replicates relative to each other were calculated using parallel line assay analysis. The geometric coefficient of variance (GCV) within assay was found to be 2.8%. Further validation of the desorption and binding activities for different vaccine products was carried out using one selected batch from each vaccine type. The reproducibility of the assay was assessed by 4-6 replicated independent assays performed on separate days. Their relative potencies were calculated using parallel line assay as described in Section 2.8.9 with the exception that PT 90/518 was used as reference. The GCV for these assays ranged from 13.5 to 22.5% (Table 3.4) for the inter-assay variation. The different vaccine products showed differences in their binding activities.

Table 3.4. Assay reproducibility and validation of A3-capture ELISA for PT eluted from different DTaP preparations by tri-sodium citrate

Vaccine	Potency /geometric mean (Range at 95% CI)	GSD	GCV (%)	*n*
DTaP-A	0.66 (0.49-0.83)	1.13	13.5	5
DTaP-B	0.45 (0.31-0.59)	1.18	17.5	5
DTaP-C	0.25 (0.19-0.31)	1.15	15.4	6
DTaP-D	0.36 (0.35-0.37)	1.15	14.8	5
DTaP-E	0.08 (0.06-0.1)	1.18	17.5	5
DTaP-F	0.09 (0.09-0.13)	1.23	22.5	4
DTaP-I	0.35 (0.21-0.49)	1.21	20.6	5

The values were analysed by parallel line assay as described in Chapter 2, Section 2.8.9. with the exception that PT was used as a reference. GSD, geometric standard deviation; GCV, geometric coefficient of variance; *n*, number of experiments.

3.3 Exploring the potential application of the developed *in vitro* assays

3.3.1 Comparison of enzymatic and binding activities of PT in vaccine formulations and in purified native PT

As defined in Materials and Methods (Chapter 2, Sections 2.7.3 and 2.8.9) the results of ADP-ribosylation activity or binding activity per SHD of test vaccine were expressed as E-units or B-units, where one E-unit or B-unit is equivalent to the activities produced by 1 μg of PT 90/518 respectively. Further comparison of A- and B- subunit activities were made between PTd in vaccine and purified PT preparation 90/518 at equal protein mass (the activities produced by 1 μg of PTd protein antigen in a vaccine product was compared to the activity produced by 1 μg of native PT). For example, for a vaccine which has a 25 μg PTd antigen content, the activities produced by 1 μg PTd in the vaccine were calculated by dividing the E- and B-units produced per SHD by 25 (Chapter 2, Table 2.2). Using DTaP-A (n=9) as an example, the mean ADP-ribosylation and binding activities were 0.06 ± 0.02 E-unit/μg PTd and 0.006 ± 0.001 B unit/μg PTd in comparison to 1 E-unit or B-unit/μg in purified PT 90/518 respectively. Thus, a large reduction in the enzymatic and binding activities of PTd antigen was evident. This indicates that the detoxification process affected both A- and B- subunit activities of PT. Since the decrease in B-subunit activity was approximately 150-fold and that of the A-subunit was 17-fold, the results indicate that the detoxification process affected the B-subunit activity more than the A-subunit activity. Similar observations were also made for other vaccine formulations tested (results not shown).

3.3.2 Comparison of different detoxification methods

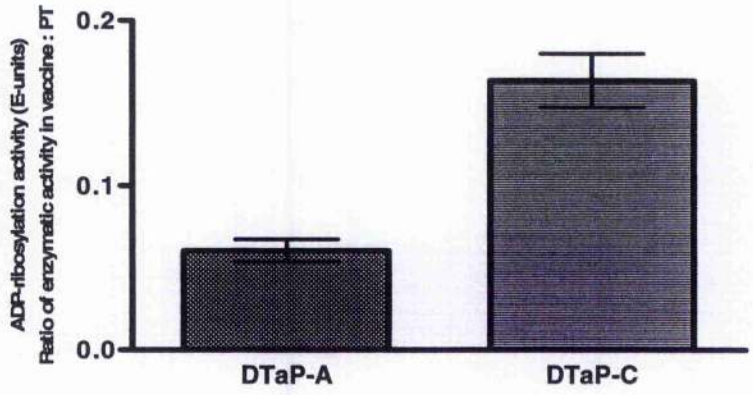
The *in vitro* assays developed can be used to determine the degree of detoxification in vaccines. Interestingly, formaldehyde-glutaraldehyde treated PT (DTaP-A; $Al(OH)_3$) significantly ($P<0.05$) retained less enzymatic activity (0.06 ± 0.02 E-unit/μg PTd, n=9) than glutaraldehyde-only treated PT (DTaP-C; $AlPO_4$, 0.17 ± 0.04 E-unit/μg PTd, n=5 Figure 3.15). On the contrary, there was significantly ($P<0.05$) less PT binding activity remaining when PT was chemically-inactivated with glutaraldehyde only (0.003 ± 0.0005 B-unit/μg PTd) rather than with formaldehyde-glutaraldehyde (0.006 ± 0.001 B unit/μg PTd, Figure 3.15).

3.3.3 Comparison of different formulations

For confidential reasons the exact compositions of the different vaccine formulations cannot be indicated in this thesis. For distinguishing different vaccine formulations, DTaP in combination with other antigen components are indicated by the letter x in this section. For example, a comparison was made between products DTaP-C and DTaP-D (x). These products were compared since DTaP-C is an intermediate bulk to the DTaP-D vaccines but the latter product was formulated with other additional antigen components. Interestingly, four batches of DTaP-D (x) products but not the four corresponding DTaP-C products showed reactivity in the HIST. One batch of DTaP-D (x), which was a clinical trial lot, showed no HIST reactivity (Figure 3.16).

Further investigation was carried out to determine whether the results seen in HIST were due to changes in PT activity by assessing the ADP-ribosylation and binding activities of PT in these two vaccine formulations. Comparison of ADP-ribosylation

(a)



(b)

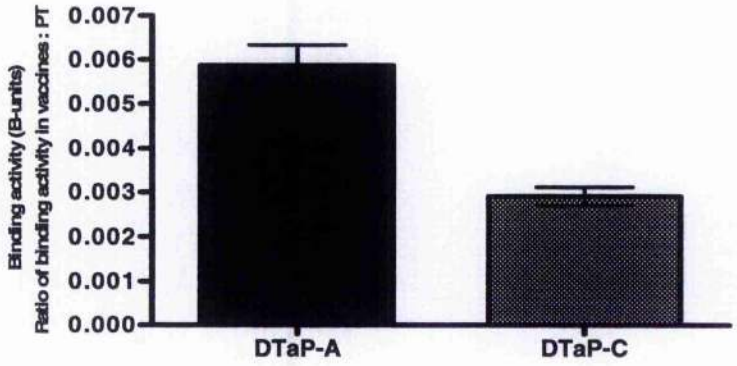


Figure 3.15 ADP-ribosylation (a) and A3 binding (b) activities of PT in vaccines compared to purified PT. The data shown represent the means of 9 lots of DTaP-A products and 5 lots of DTaP-C products. 1 E-unit and B-unit is equivalent to the activity produced by 1 μg PT 90/518. The activity produced by 1 μg PT protein in vaccines was calculated by dividing the activity per SHD of vaccine by the nominal PTd content in the vaccine (Chapter 2, Table 2.2)

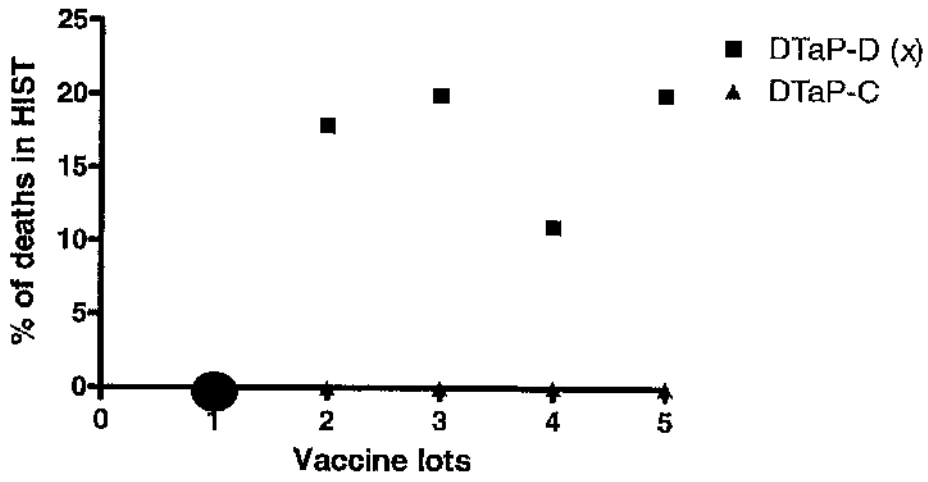
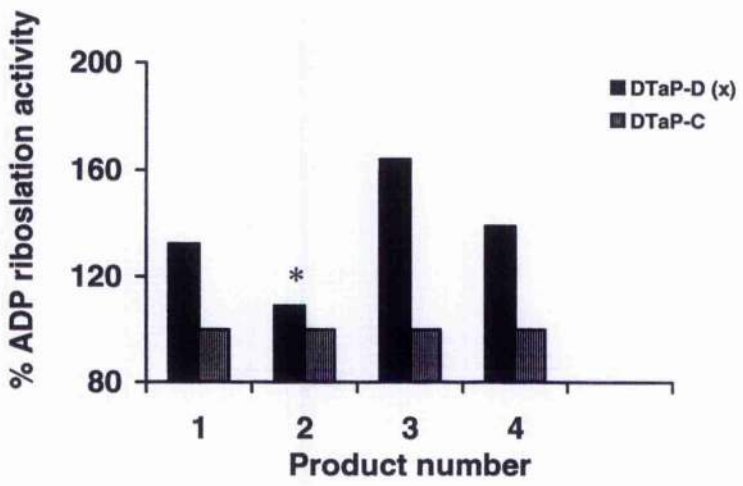


Figure 3.16. HIST results for DTaP-D (x) and corresponding batches of DTaP-C. All DTaP-D (x) products showed histamine reactivity (mouse deaths) in the HIST with the exception of one batch highlighted (red). This was a clinical trial lot, and showed no reactivity in the HIST even at 2 SHDs.

activity and binding activity in DTaP-D (x) is shown in Figure 3.17a, b. The activity of the corresponding DTaP-C was taken as 100% for comparative purposes. The binding activities of PT/d in these vaccine products were determined using Fet-capture ELISA. For these experiments vaccine samples were treated with EDTA to release the PT from the $AlPO_4$. To calculate the binding activity using 90/518 as a reference for both products, a standard-curve was constructed using the optical densities corresponding to the various concentrations of PT 90/518. Binding activity in the supernatant was calculated against the standard-curve. Further comparisons were also made of the clinical trial batch and production batches on the mathematical ratio of binding (B) activity over enzymatic activity and is presented in Figure 3.18.

It was noted that the use of PT 90/518 as a common reference to compare the binding activity in different vaccine formulations may not be appropriate, since this does not take account of the desorption differences between different vaccine formulations. Since the E-HPLC method can be used to determine the enzymatic activity of PT even when it was adsorbed to the adjuvant, the enzymatic activity found in vaccines would reflect the total amount of PT present in the vaccine formulation. Therefore the PT ADP-ribosylation activity in the supernatant after desorption was used in comparison to the total activity in the un-desorbed sample to work out the percentage of desorption and then to calculate the total binding activity in test sample, e.g. if the binding activity in supernatant is 5 which accounts for 20% of total PT in the supernatant, then the binding activity would be 5x5 (100%) to give total binding activity in the vaccine. The results showed that both enzymatic and binding activities of these products were higher in DTaP-D (x) products than their corresponding DTaP-C products (Figure 3.17). It was also found that the increase in both activities was not due to the addition of other

(a)



(b)

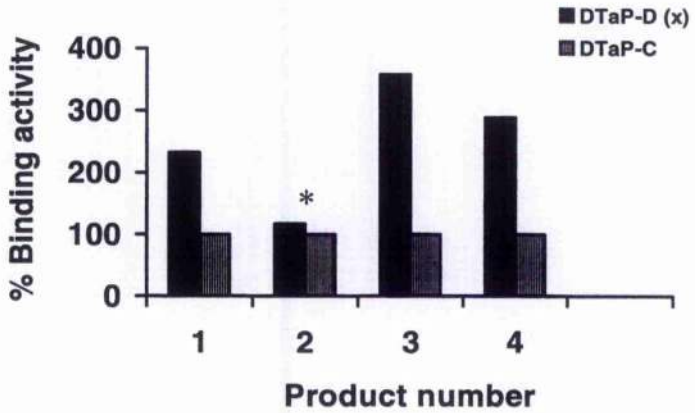


Figure 3.17. Comparison of the (a) ADP-ribosylation and (b) binding activities of PT present in DTaP-D (x) and their corresponding batches of DTaP-C vaccine products. * Clinical trial lot and its intermediate bulk. For binding, only the eluted PT released into the supernatant can be assessed hence the total binding activity as shown in b was calculated by determining the PT ADP-ribosylation activity in the supernatant in comparison to the total activity in the sample using ADP-ribosylation assay. This was used to calculate the total binding activity in test sample, e.g. if the binding activity in supernatant is 5 which counts for 20% of total PT in the supernatant, (as determined by the ADP-ribosylation method), then the binding activity would be 5x5 (100%) to give total binding activity. For comparative purposes, the binding activity of DTaP-C product was taken as 100 percent.

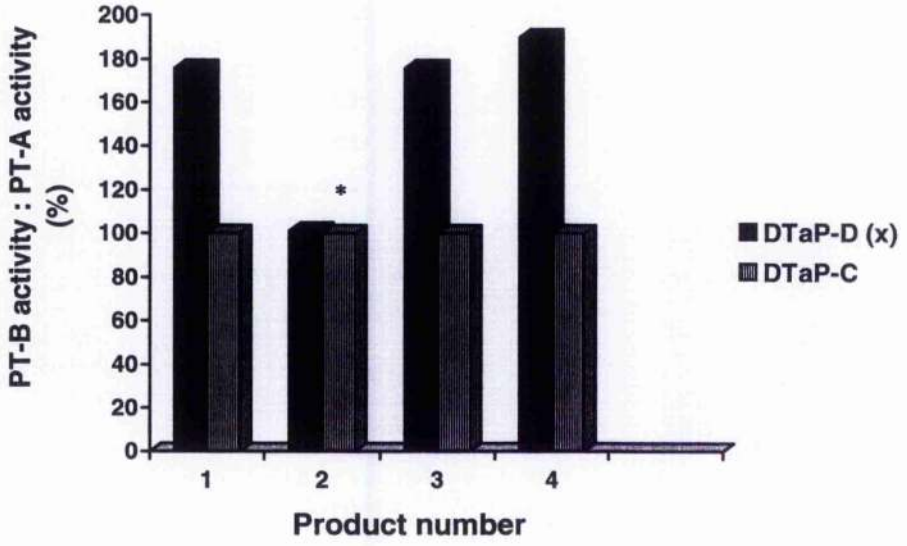


Figure 3.18. Comparison of ratio of PT-B activity to PT-A activity in the vaccines. * Clinical trial lot. For comparative purposes, the PT- B activity divided by PT-A activity for DTaP-C was taken as 100 percent.

vaccine components (x), since addition of x to DTaP-C did not alter enzymatic activity or binding activity (results not shown). It is noteworthy that the increased binding activity in DTaP-D (x) (100-250%) relative to DTaP-C, was much higher than the increase in enzymatic activities (30-60%, Figure 3.17). In addition, the ADP-ribosylation activities or the binding activities of the clinical trial lot vaccines and its intermediate bulk, were similar and the clinical trial lot displayed no reactivity in the *in vivo* HIST (Figure 3.17). The PT-B activity divided by PT-A activity of DTaP-C was taken as 100%. Interestingly, it was found that the three batches of DTaP-D (x) with the exception of the clinical trial lot batch showed a higher activity (Figure 3.18).

3.3.3.1 Preliminary investigation of the effect of Tween-80 on enzymatic and binding activities of vaccines

On closer examination of the procedures involved in the preparation of the DTaP-D (x) it was noticed that Tween-80 was used as a detergent in the formulation. The effect of Tween-80 on PT was assessed by the ADP-ribosylation assay. DTaP-C was treated with different concentrations of Tween-80 and DTaP-C treated with PBS was used as the control. All samples were kept at 4^0C until assay. The results are presented in Figure 3.19. Increased ADP-ribosylation activity was found as the Tween-80 concentration was increased, up to 0.1%. The enhancement was rapid and sustained over a period of time (Figure 3.20).

To investigate if this effect was product specific, two pertussis toxoid preparations and four other DTaP products and one whole-cell pertussis vaccine (DTPw-J) were also included in the assay. All samples were treated with Tween-80 at 0.1% final

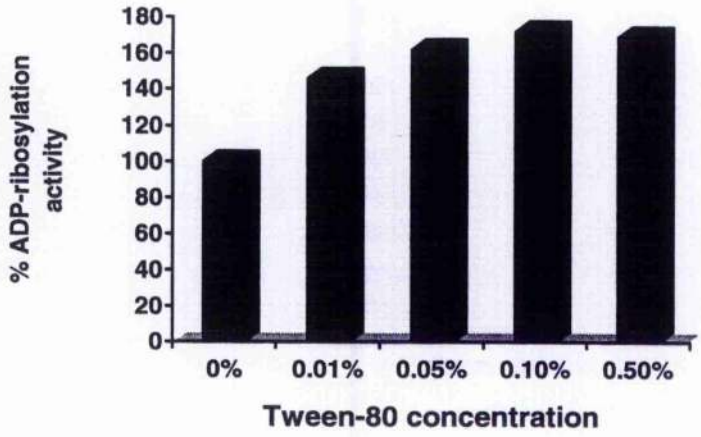


Figure 3.19. Effect of concentrations of Tween-80 on ADP-ribosylation activity in DTaP-C. The enzymatic activity in the vaccine preparation treated with PBS was taken as 100%.

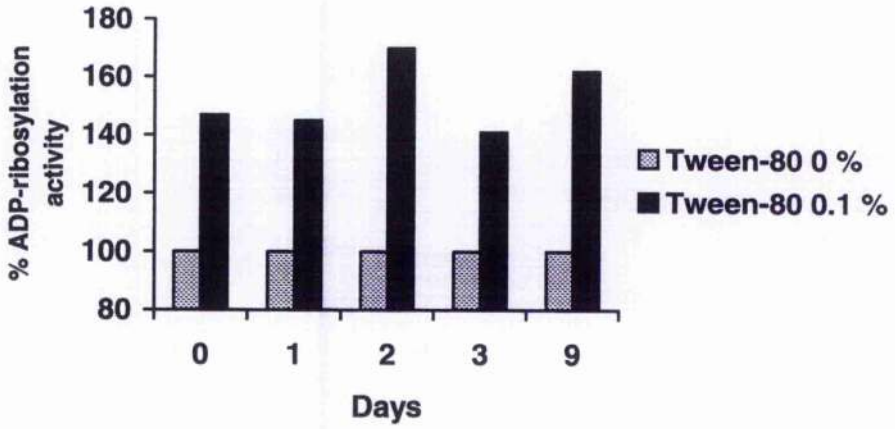


Figure 3.20. Time course of Tween-80 effect on DTaP-C. For control, the enzymatic activity of DTaP-C product treated with PBS was taken as 100%.

concentration and assayed for ADP-ribosylation activity. The results are shown in Figure 3.21. The effect of Tween-80 appeared to be product-specific as both pertussis toxoid preparations and other pertussis-based vaccines from other manufacturers did not show markedly enhanced enzymatic activities after the treatment with Tween-80 in comparison with DTaP-C (DTaP-4, Figure 3.21). However this was not the case for binding activity. Furthermore the *in vivo* histamine sensitisation experiments also did not show differences between the Tween-80 treated and the untreated samples (results not shown).

The finding that Tween-80 enhanced ADP-ribosylation activity but not binding activity in a product dependent manner was also confirmed using monoclonal antibody-capture ELISA (Chapter 2, Section 2.9). The monoclonal antibodies specific to the S1 subunit and S2/S3 subunits were used to investigate their subunit specific immuno-reactivity to PT/d present in DTaP-A and DTaP-C products. The two vaccine products were treated with or without Tween-80 and the reaction with monoclonal antibody to S1 or monoclonal antibody to S2/3 are shown in Figure 3.22. The results demonstrate that the available binding of the antibody to the S1 subunit increased following treatment of DTaP-C with Tween-80 whereas negligible changes were observed in the binding of the antibody to the S2/3 subunit for both vaccines.

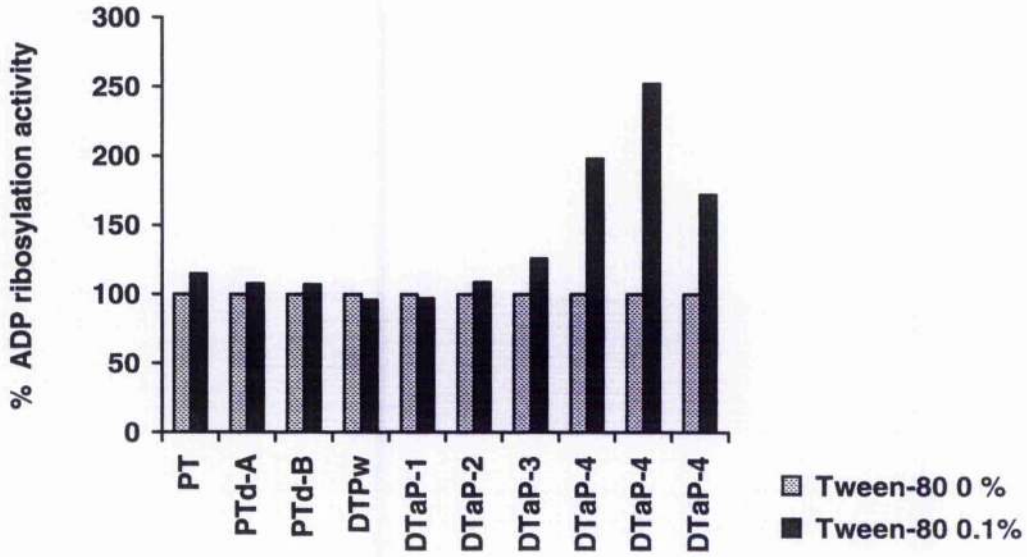
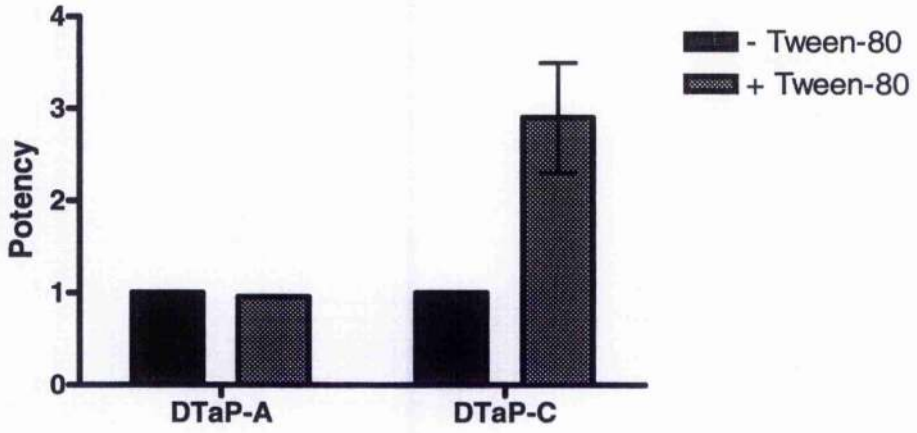


Figure 3.21. Effect of Tween-80 on different vaccine products. 1, 2, 3, 4 represent different manufacturers. The enzymatic activity in the vaccine preparation treated with PBS (0% Tween-80) was taken as 100%.

(a)



(b)

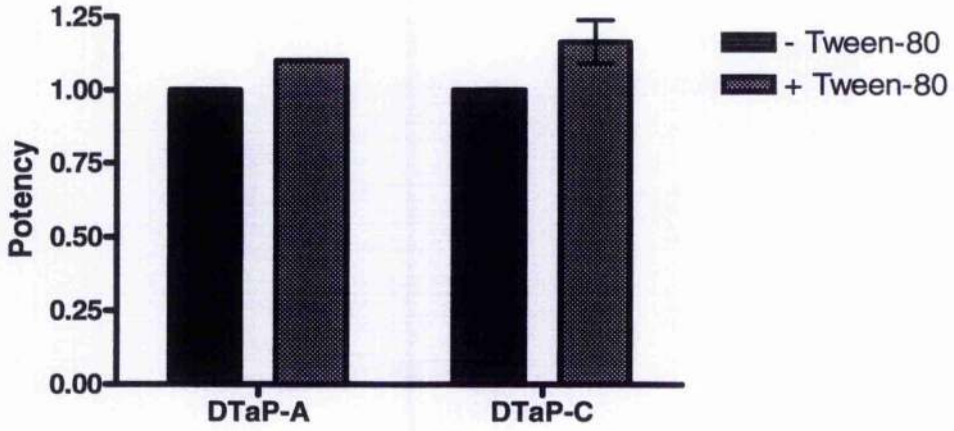


Figure 3.22. Comparison of the binding of monoclonal antibodies to S1 and S2/3 to DTaP-A and DTaP-C products in the presence and absence of 0.1% Tween-80. (a) anti-S1 monoclonal (b) anti-S2/3 monoclonal. The values were analysed by parallel line assay as described in section 2.8.9 with the exception that DTaP without treatment was used as reference and given a potency value of 1. DTaP-C results were the average of two experiments

3.3.4 Relationship of activities of A- and B- subunits of PT/d in vaccines and the *in vivo* HIST

3.3.4.1 Role of A- and B- subunit activities in vaccines and in the HIST

It is generally believed that the B-subunit facilitates the binding of PT to host cell receptors, which is then followed by the internalisation of the toxin and translocation of the S1 subunit into the cytosol, where the S1 subunit can then exert its toxic effects by catalysing the ADP-ribosylation of G proteins. Therefore, both A- and B- subunit activities of PT are thought to contribute to the toxicity observed *in vivo* by the HIST. For example, DTaP-I products displayed high binding activity (0.26 B unit/SHD, n=2) but did not show reactivity in HIST since the enzymatic active site has been eliminated by mutation. Interestingly DTaP-D, DTaP-E and DTaP-F products (Chapter 2, Table 2.1), which were all identical in terms of the detoxification reagent used (glutaraldehyde), adjuvant type and antigen composition (with the exception of DTaP-E), had different behaviours in the HIST. DTaP-D products, but not the other two formulations, showed reactivity in the HIST (Figure 3.23c). DTaP-E was an intermediate bulk to DTaP-F and contained other vaccine antigens. Further investigations were carried out to compare the A- and B- subunit activities in these vaccine products to explore the relative roles of the enzymatic and binding activities in relation to the HIST. Binding was performed using A3-capture ELISA. The results are shown in Figure 3.23 a,b. The mean enzymatic activity in all products was not statistically different ($p>0.05$). In contrast, the mean A3-binding activity of DTaP-D was found to be significantly higher ($P<0.05$) in comparison to DTaP-E and F vaccines (Figure 3.23b). This suggests that the higher binding activity may, to some extent,

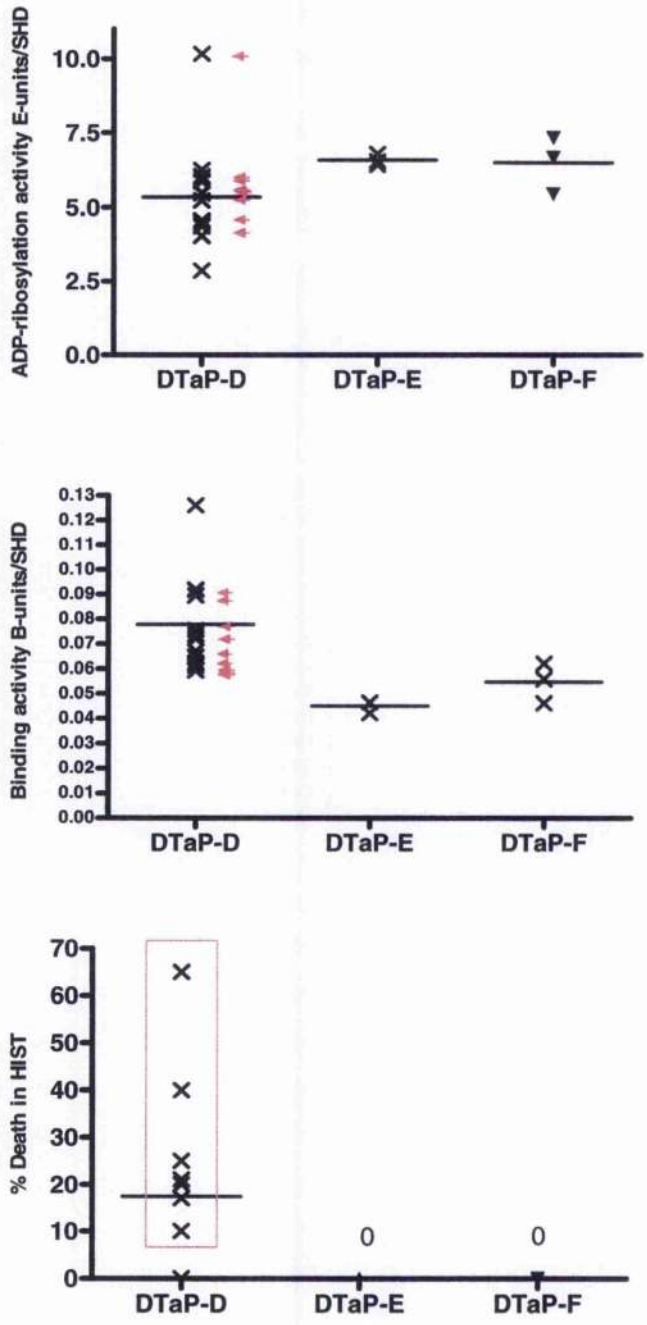


Figure 3.23 Comparison of PT A- and B- subunit activities and the *in vivo* toxic activity using HIST in DTaP-D, E and F vaccines. The data shown represent the means of at least 12 lots of DTaP-D products and 3 lots each of DTaP-E and F products. Red indicates reactive batches. The non-reactive A- and B- subunit activities range from 2.7-6.25 and 0.06-0.13 E-unit and B-units respectively. 1 E-unit and B-units is equivalent to the activity produced by 1 μg PT 90/518.

explain the higher reactivity of these products observed *in vivo*. It is possible that there may be a critical level of each of the A- and B- subunit activities that needs to be present in order to be reactive in the HIST.

3.3.4.2 Preliminary interpretation of the relationship between *in vitro* and *in vivo* tests of PT toxicity

Although the experimental data (Table 3.5), showed that, in general, there was no apparent correlation between the higher enzymatic activities in vaccines and higher mean percentage death in HIST, the enzymatic activity of PT may still play an important role in the death observed *in vivo* in the HIST. A higher binding activity, as in the case of DTaP-D appeared to increase the *in vivo* toxic effect of the enzymatic activity presumably by aiding the accessibility of cellular targets to the PT S1 subunit (Table 3.5 and Figure 3.23). A direct correlation between *in vivo* and *in vitro* assays for different vaccine formulations could not be established using either A- or B- subunit activities alone (Figures 3.5 and 3.24). The relationship between these *in vitro* and *in vivo* activities was then initially explored using simple comparisons, for example by multiplying the A- and B-subunit activities (Figure 3.24) or by adding or dividing the A- and B-subunit activities. However a clear-cut meaningful relationship between the *in vitro* and *in vivo* systems could not be found, either for the different vaccine formulations or for different lots within the same type of product. For example, for DTaP-D products, the A- x B-subunit activities of the lowest and highest HIST reactive batches were 0.23 and 0.55 respectively, whereas the A- x B-subunit activities of the lowest and highest HIST non-reactive batches were 0.25 and 0.45, which were very similar. Likewise when relating the data by adding or dividing the A- and B-subunit activities no relationship could be established (results not shown).

Table 3.5 Comparison of *in vitro* and *in vivo* activities for different pertussis-based combination vaccines

	DTaP-A *n*=9	**DTaP-C** *n* =5	**DTaP-D** *n* =12	**DTaP-E** *n* =3	**DTaP-F** *n* =3
***Histamine reactivity**	5 ± 6	5 ± 7	17 ± 19	0	0
E-units/ SHD	1.52 ± 0.52	3.20 ± 0.98	4.89 ± 1.0	6.58 ± 0.12	6.5 ± 0.96
B-units/ SHD	0.15 ± 0.034	0.06 ± 0.003	0.08 ± 0.019	0.05 ± 0.003	0.05 ± 0.008

* % death. All values are expressed as mean and standard deviations of the mean. 1 E-unit and B-unit is equivalent to the activity produced by 1 µg PT 90/518.

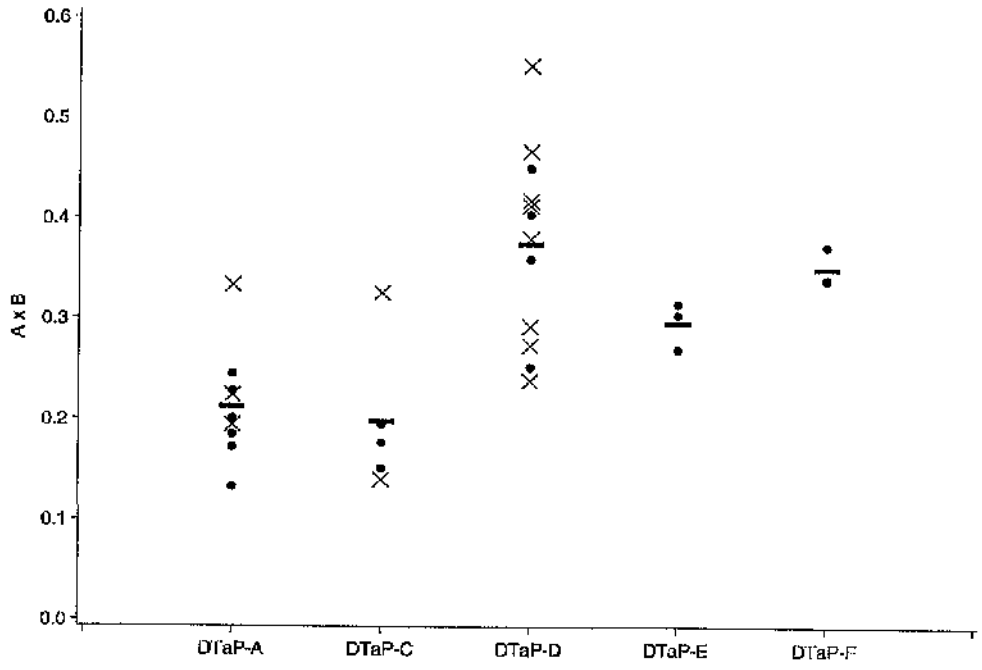


Figure 3.24. A- x B-subunit activities in pertussis vaccines. The average value per group of vaccines is represented by a '-', crosses indicate vaccine batches that show reactivity in the *in vivo* HIST and dots were HIST non-reactive batches. AxB was calculated by multiplying the ADP-ribosylation activity units (E-units) per SHD of test vaccine by the binding activity units (B-units) per SHD vaccine.

To further explore the possible relationship between histamine reactivity and enzymatic and binding activities, the following mathematical equation [1] was used according to the method described by the Heinemann working group (Chapter 2, Solving equations and inequalities, 2002). Because the number of batches tested in the present study was very limited, the analysis in this section can only be interpreted as preliminary.

$$\text{Death} = A\,(H \times B) + C \qquad [1]$$

where, A is an associated parameter for E-HPLC (H) and binding (B) activity and C is a constant parameter. Using the data generated from PT preparation 90/518, the equation was constructed as explained in Chapter 2, Section 2.9.2 and is shown below:

$$\text{Death} = 1.77\,(H \times B) + 14.4 \qquad [4]$$

The experimental data fitted well with the equation and the results are shown in Table 3.6. This equation was then further used to test its suitability for assessing residual PT activity in pertussis vaccines and was applied to vaccine formulations A, C and D. For these products, the number of batches tested in the *in vivo* and *in vitro* assays were ≥ 5. Because the A- and B- subunit activities varied between products, according to experimental data, the parameters for each product used in the equation would be product dependent. Using DTaP-A products as an example, the A and C values can be calculated using equation [1].

Table 3.6 Comparison of actual deaths (shown in brackets) due to purified PT preparation 90/518 in HIST and predicted deaths from enzymatic and binding data using equation 4.

PT	16.7 ng			50 ng		
	*Actual HPLC	**Actual binding	Predicted death (%)	*Actual HPLC	**Actual binding	Predicted death (%)
	16.87	0.16	19.22	101.0	0.32	71.46
	17.59	0.25	22.26	52.65	0.50	60.98
	29.15	0.29	29.36	67.43	0.31	50.98
	22.39	0.14	19.98	70.22	0.46	71.69
	23.46	0.23	23.78	-	-	-
Mean	21.89	0.21	23 ($^{+}$23)	72.83	0.40	64 ($^{+}$70)

*Enzymatic activities of 90/518 were expressed as described in Chapter 2, section 2.7.3. **Binding values refer to OD values. $^{+}$ values shown are the mean actual deaths observed for 16.7 ng (n=13) and 50 ng (n= 30) PT, the raw data values are shown in Appendix E.1.

Group 1: Higher mean death, enzymatic and binding activities of 10, 2.33 and 0.585 respectively (Appendix E.2). Group 2: lower mean death, enzymatic and binding activities of 0, 1.252 and 0.816 respectively (Appendix E.2). Thus using the mathematical model two equations can be generated using these two groups:

$$10 = A\ (2.33 \times 0.585) + C \qquad \text{group 1}$$

$$0 = A\ (1.252 \times 0.816) + C \qquad \text{group 2}$$

The mathematical equation for DTaP-A products can now be represented as follows:

$$\text{death} = 29.3\ (H \times B) - 29.9$$

When the death equals zero then the equation for enzymatic and binding activity for DTaP-A can be represented as follows:

$$H \times B > 1.02$$

The enzymatic and binding activities of all the DTaP-A products were compared to the death threshold line. A similar procedure was carried out for DTaP-C and DTaP-D products and the death threshold line was deduced to be $H \times B > 1.08$ and $H \times B > 3.22$ respectively. The ADP-ribosylation and binding activities in relation to this equation line that represents no reactivity *in vivo* is shown in Figures 3.25, 3.26 and 3.27 for DTaP-A, C and D respectively. Experimental data showed that when enzymatic activity was below a certain value, there was no death observed regardless of binding activity. Therefore, a E-HPLC-death threshold (E-HPLC cut-off) for each product was defined as the minimal level of ADP-ribosylation activity required for showing any reactivity in

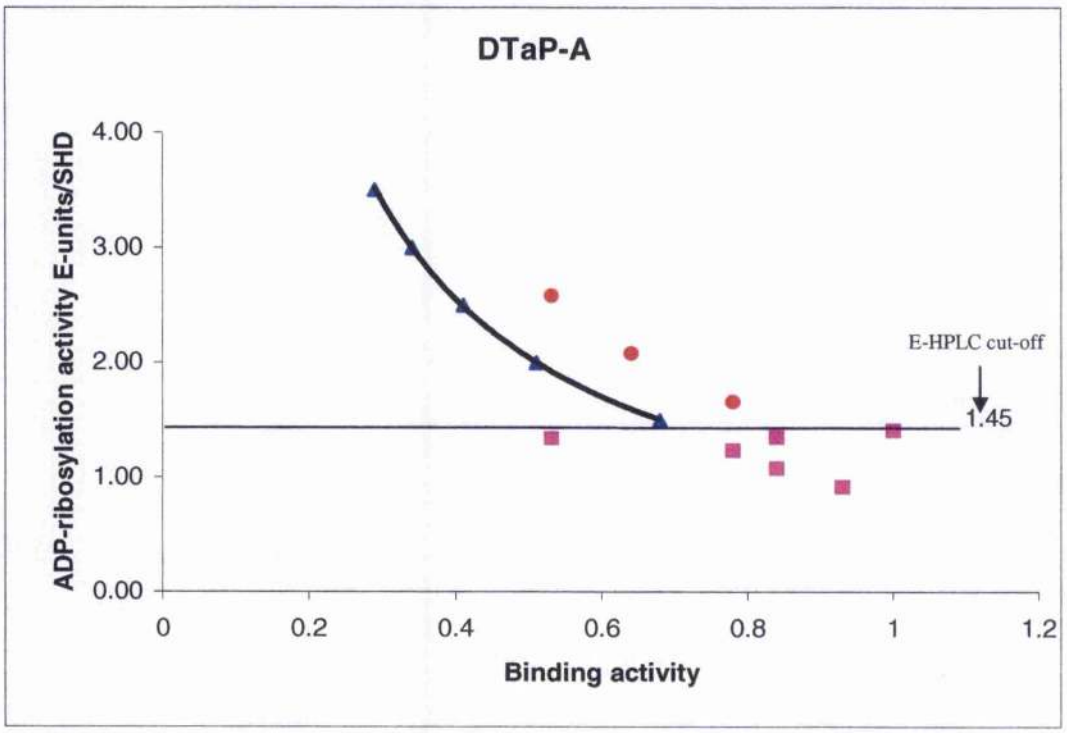


Figure 3.25. Relationship between enzymatic activity, binding activity and HIST reactivity for DTaP-A (n=9) products. Red dots indicate reactive batches in HIST. Pink squares indicate HIST non-reactive batches. For binding, potency values were analysed by parallel line assay using a reference vaccine as described in Chapter 2, section 2.8.9. The equation line (blue triangles) corresponds to the death threshold. The horizontal line indicates E-HPLC cut-off value.

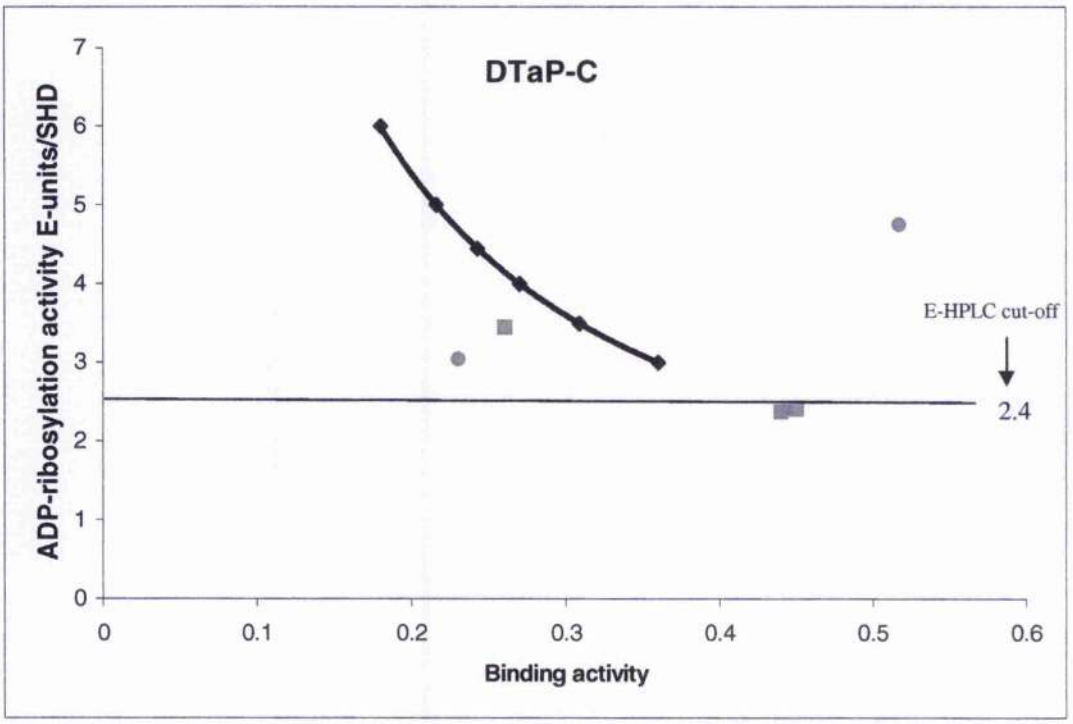


Figure 3.26. Relationship between enzymatic activity, binding activity and HIST reactivity for DTaP-C (n= 5) products. Red dots indicate reactive batches in HIST. Pink squares indicate HIST non-reactive batches. For binding, potency values were analysed by parallel line assay using a reference vaccine as described in Chapter 2, section 2.8.9. The equation line (blue triangles) corresponds to the death threshold. The horizontal line indicates E-HPLC cut-off value.

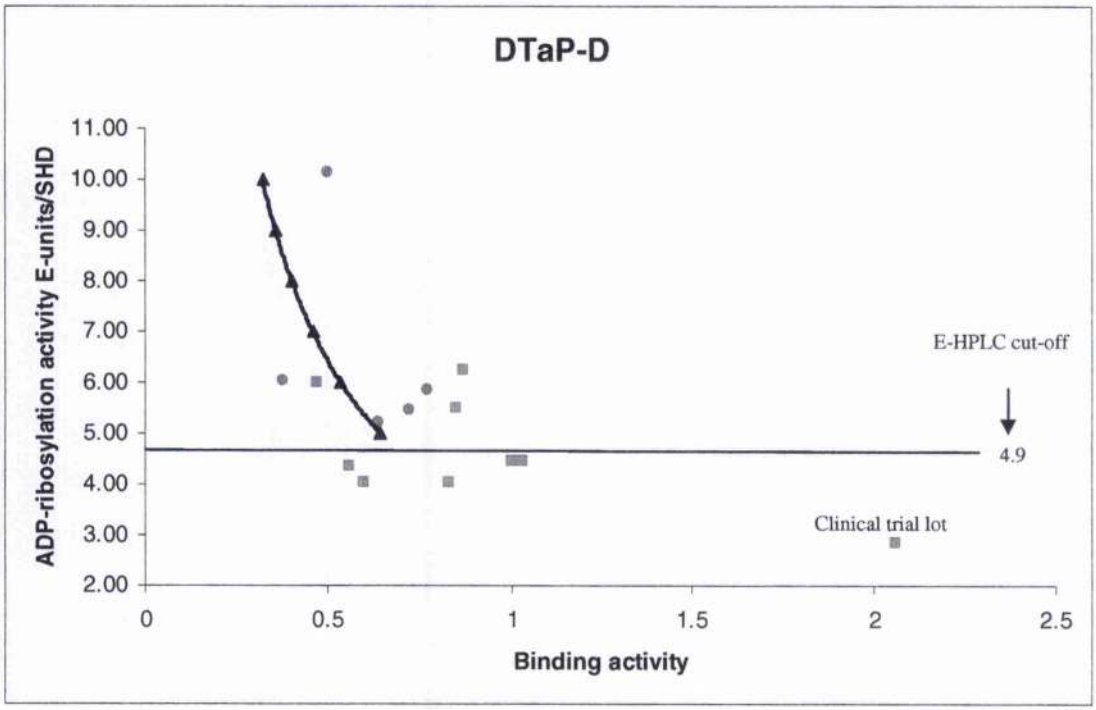


Figure 3.27. Relationship between enzymatic activity, binding activity and HIST reactivity for DTaP-D (n=14) products. Red dots indicate reactive batches in HIST. Pink squares indicate HIST non-reactive batches. For binding, potency values were analysed by parallel line assay using a reference vaccine as described in Chapter 2, section 2.8.9. The equation line (blue triangles) corresponds to the death threshold. The horizontal line indicates E-HPLC cut-off value.

the HIST. This was calculated by dividing the death threshold value (e.g. for DTaP-A, this is 1.02) by average binding activity of the two groups used to create the death equation line for DTaP-A (0.70).

The A- and B- subunit activities in all the reactive lots (3) of the test vaccines of DTaP-A type were found to be greater than the death threshold line and also the HPLC cut-off line indicating a clear positive relationship between the *in vitro* and *in vivo* methods. Using the same parameters (E-HPLC cut-off line and death threshold line) specific for DTaP-C products, the activities of 2/3 batches in the *in vivo* HIST were correctly identified as negatives. However, from the two reactive products only one was positively identified and the other was a false negative, i.e. the product showed reactivity in the HIST but was below the death threshold line. A total of 14 batches of DTaP-D products were tested. Five batches showed reactivity in HIST. Reactive lots for this product were defined by comparison with the activities of a reference vaccine which had been shown to be clinically safe. 4/5 of the reactive products were positively identified, that is higher than the death threshold line and also the E-HPLC cut-off. On the other hand, two batches were false positives and one batch was a false negative. The reasons for the discrepancies could be caused by the large variation in the HIST. It has been shown that the HIST is extremely variable making the validation of any new method against it very difficult (van de Kappelle *et al.*, 1997; Xing *et al.*, 2002). Using the proposed mathematical model for histamine reactivity, there appears to be a product-specific minimal level of enzymatic activity required (i.e. E-HPLC cut-off value) for displaying reactivity *in vivo* in all three products. The binding activity only contributes to reactivity *in vivo* when the E-HPLC activity is above the cut-off value.

Chapter 4

Discussion

4.1 Standardisation and assessment of residual PT ADP-ribosyl transferase activity in pertussis vaccines and its relationship with the *in vivo* HIST

WCV and ACVs play an essential role in controlling the global burden of the pertussis disease and in these vaccine preparations, PT, in its detoxified form, is considered to be an essential component for protection against the disease. The detoxification process needs to be carefully controlled so that the protective epitopes are preserved whilst minimising toxicity levels. Since PT has a number of toxic activities *in vivo*, monitoring the residual PT activity and any reversion of toxoid to toxicity in pertussis vaccines is essential if safe and effective immunisation programs are to be maintained. Currently the only toxicity test practical and recommended by regulatory authorities for this purpose is the *in vivo* HIST (WHO, 1998; EP, 2006). However, this test is a lethal challenge test and also suffers from large intra/inter laboratory variations (van de Kappelle *et al.*, 1997; Xing *et al.*, 2002). Therefore a replacement test is needed. Although the modified *in vitro* ADP-ribosylation assay was shown to directly correlate with the *in vivo* HIST when using native PT preparations (Cyr *et al.*, 2001; Yuen *et al.*, 2002), these studies did not address the relationship between ADP-ribosylation activity in pertussis vaccines and reactivity in the HIST. In the present study, the ADP-ribosylation assay has been further optimised and standardised. To assess the possibility of replacing HIST with the ADP-ribosylation assay, the relationship between the PT enzymatic activities in various vaccine formulations and their reactivity in the HIST has been investigated.

4.1.1 Optimisation and standardisation of the ADP-ribosylation assay

The ADP-ribosylation activity of PT was found to be higher when using the substrate without the linker (FAC-$G_{\alpha i3}$C20) in comparison with that FAC-$(CH_2)_5$-$G_{\alpha i3}$C20 used in

previous studies (Cyr *et al.*, 2001; Yuen *et al.*, 2002). From this result, the substrate without the linker was chosen for the assay, as it would increase its sensitivity (Chapter 3, Figure 3.3). The reason for this finding is not known but it can be speculated that the one with the linker might be less soluble and hence less reactive with the enzyme, or it might be more hydrophobic and aggregate more, and hence become less reactive. Molecular modelling and nuclear magnetic resonance studies could be used to investigate these possibilities but these experiments were beyond the scope of the present study.

Other than PT, there is a number of other antigen components present in WCV and ACV preparations. In addition, $Al(OH)_3$ and $AlPO_4$ are used as adjuvants for WCVs and ACVs. For this reason, the effect that the different antigens and these adjuvants might have on the assay system was investigated. In agreement with previous studies, the ADP-ribosylation assay proved to be specific for PT as none of the other vaccine antigen components demonstrated ADP-ribosyl transferase activity including PRP-T, which has not been previously assessed and is now a component present in the newer multivalent vaccines (Halsey, 2001). In contrast, in this study, the adjuvants commonly used in pertussis vaccines demonstrated a differential effect on PT ADP-ribosylation activity. The presence of $Al(OH)_3$ but not $AlPO_4$ decreased the ADP-ribosyl transferase activity of PT. This may possibly explain the lower slopes observed in some selected vaccine products when using the standard addition method where vaccine was used as matrix for reference PT preparation when constructing the standard curve (Cyr *et al.*, 2001). The reason for this is unknown and Yuen *et al.* (2002), using a similar approach, reported that the adjuvant did not interfere with the enzymatic activity. Since the aluminium salt effect in this study occurred using either substrate with or without the $-(CH_2)_5-$ linker, and the only difference between this study and the previous study was

the batch of substrate, the observed effect may have been be due to unidentified impurities present in this batch of substrate, generated during the synthesis and purification. There have been occasions when the supplied substrates were rejected due to the unexpectedly low reactivity with the enzyme (C-T Yuen, personal communication). Therefore, in view of this effect of adjuvant on the ADP-ribosylation activity of PT, it was decided to add the appropriate aluminium salt to the PT preparation 90/518 when preparing the standard-curve, to compensate for the effect that it had on PT activity. Thus the residual PT enzyme activity of test vaccines containing different adjuvants was calculated from the appropriate standard-curves.

In the present study, due to occasional reagent variability, there were some slight inconsistencies in the day-to-day measurements of ADP-ribosylation activities in vaccine products. To reduce such variations, the assay was standardised by including a positive control vaccine, Biken, into each assay and a validity criterion was established using the ratio of ADP-ribosylation activities of PT to ADP-ribosylation activities of Biken (Chapter 3 section 3.1.2).

4.1.2 Residual PT ADP-ribosylation activities in different pertussis vaccine formulations

In agreement with previous observations, no ADP-ribosylation activity was observed when using DTaP-I, which contained a genetically-inactive enzyme subunit (Chapter 3, Table 3.2). The residual PT enzymatic activity in WCVs (DTPw-J) was also found to be much lower in comparison to ACVs, with the exception of DTaP-G and this was similar to the findings of Cyr *et al.* (2001). These authors suggested that the low enzyme activity of DTPw might be due to the degradation of the synthetic substrate by unheated

whole-cell components such as proteases. However, this was not observed in this study since degradation of the substrate was not seen. The reason for the observed lower enzymatic activity found in these vaccines is unclear. The DTPw products were not subjected to HIST in the present study because WCV composition is much more complex and varied, containing large amounts of LPS and other cellular components which could also affect the outcome of HIST. The products DTaP-G and DTaP-H that showed low enzyme activities and also did not cause histamine sensitisation in mice, were booster vaccine formulations corresponding to the full dosage vaccine DTaP-C and their PTd antigen content was lower than that of DTaP-C. The result for these two booster vaccines may indicate that the present ADP-ribosylation assay could be applied quantitatively and specifically to vaccines of similar formulations.

Apart from DTaP-I and products where only 1 or 2 batches were tested in this study, batch-to-batch variations of ADP-ribosylation activity were observed for all vaccine formulations. This within-product variability may be partly explained by the fact that both WCVs and ACVs are produced from a living organism in a batch-wise procedure. Subtle changes in materials, in the process itself or conditions such as pH, availability of reactive amino acids in the protein (PT) during the chemical inactivation reaction, can affect the extent of detoxification and type of chemical modification (Metz *et al.*, 2004). Apart from variability within products, different formulations showed differences in their enzymatic activities ranging from 0-10.2 E-units per SHD. Therefore a common level of enzymatic activity of PT for all vaccine formulations could not be defined. Furthermore, the enzymatic activities did not correspond to their total PT protein concentration as specified in the vaccine formulations, e.g. the PT antigen contents of DTaP-A and B were reported to be 20% higher than those of DTaP-C to F, but the enzymatic activity was much lower in the former products. Based on the

observation that antigens other than PT do not have any intrinsic ADP-ribosyl transferase activity, the variation in enzyme activities was likely to be as a result of the different detoxification methods used in the production procedures because products DTaP-A and B were from one manufacturer while products DTaP-C to F were from another (Section 4.3.1). Chemical treatment has been widely used by manufacturers to detoxify PT. Different detoxification procedures using different reagents/or conditions have been shown to result in different amino acid side-chain modifications and changes in conformational and epitope binding patterns for the resulting PTd (Burns *et al.*, 1987; Ibsen, 1996). The mechanisms and the sites of different toxoiding reactions on the PT molecule have not yet been defined. Thus, the PTd present in pertussis vaccines from different manufacturers could be modified at different sites of the A-subunit, B-oligomer or both and this deserves further investigation.

4.1.3 Relationship between residual PT activity in vaccines and reactivity in the HIST

Product DTaP-C was the intermediate bulk to DTaP-D, which had identical DTaP components except that, in addition to these antigens, DTaP-D also contained other antigen components. Comparison of the enzymatic activities in products DTaP-C and DTaP-D showed that, in general, higher enzymatic activities were found in the latter products which also showed more reactivity in HIST (Chapter 3, Figures 3.6 and 3.17). The difference between these two products was unlikely to be caused by other vaccine components since addition of the other antigens to DTaP-C did not result in either an increase in the enzymatic activities or in the reactivity *in vivo* (DK Xing, personal communication). These data suggest that, apart from the detoxification procedure, other formulation factors might affect the residual ADP-ribosylation activity in the final

vaccine products. For example, Tween-80 is a detergent used in DTaP-D vaccine final formulation. Preliminary investigation showed that addition of Tween-80 to DTaP-C products enhanced the ADP-ribosylation activities (Section 3.3.3).

At present, the HIST is the only practicable means for detecting active PT in pertussis vaccines. HIST is a lethal test and large variations in test performance have been observed (van de Kappelle, 1997). In a more recent international collaborative study, significant differences (>30-fold) in HIST results between individual laboratories were observed, reflecting differences in animal sensitivity and other assay conditions (Xing *et al.*, 2002). Therefore it is possible that some vaccine batches which showed reactivity in the present study may not have been judged to be reactive in a test performed by other laboratories that use a different animal strain or test procedure. Moreover, the residual active PT is expressed as the proportion of animals that die upon sensitisation with a SHD of vaccine. Since this test, as done routinely, is highly variable, it would not give a quantitative indication of toxicity. For this reason, a direct quantitative comparison between the enzymatic activity and reactivity in HIST for each vaccine formulation would not be possible. Indeed, a clear positive relationship between the residual PT enzyme activity in vaccines and the *in vivo* reactivity observed by the HIST could not be found in the present study under the current experimental conditions. For example, none of the DTaP-E and F vaccines, possessing high enzyme activities, showed reactivity in HIST whereas approximately 60% of DTaP-D products, with similar or lower enzyme activities, showed reactivity in HIST (Chapter 3, Figure 3.5).

PT preparation coded 90/518 was used as the in-house reference for both enzymatic-HPLC and HIST assays in this study. This preparation has been assigned a unitage of 2100 IU per ampoule in terms of the 1st International Standard for PT (NIBSC code

JNIH-5) in HIST (Xing *et al.*, 2002). However, because the contribution of ADP-ribosylation activities in these references to the reactivity observed in HIST is uncertain, the same unitage in HIST cannot be applied to the enzyme activity. Therefore in the present study, an arbitrary E-unit was used to express the amount of ADP-ribosylation activity in one SHD of vaccine and 1 E-unit was equivalent to the enzymatic activity produced by 1 μg of PT (90/518). In the current HIST system, the 50% histamine sensitisation lethal dose (HSD_{50}) for preparation 90/518 is within the range 16.6-50 ng/mouse which corresponds to 1.75 IU-5.25 IU of bioactivity (Xing *et al.*, 2002). Interestingly, the residual enzymatic activities found per SHD of the vaccine formulations were approximately 18-200 (Chapter 3, Table 3.2 and Section 4.3.4.1) fold higher than the HSD_{50} values of PT 90/518, and with these high values of activity, all mice in the test groups would be expected to show signs of reactivity if the ADP-ribosylation activity is the sole factor in the *in vivo* HIST. However, this was not the case. Therefore, the results obtained from the study indicate that ADP-ribosylation activity in vaccines could not directly reflect the reactivity seen in HIST except in the case of DTaP-g, which does not have the enzymatic activity and also did not show any reactivity in HIST.

PT is an AB type bacterial toxin possessing two functionally distinct domains: the enzymatic A-protomer and the B-oligomer that facilitates host-cell binding and entry of the toxic enzymatic A-protomer into the cell. Although the precise mechanisms and the role of each A- or B- subunits on reactivity *in vivo* are unknown, this dual biological function of the PT molecule is likely to be fully reflected in the HIST but not in the present ADP-ribosylation assay which measures only the A-protomer activity. Different detoxification processes can yield quite distinct products (Burns *et al.*, 1987; Gupta *et al.*, 1987; Gupta *et al.*, 1987b; Ibsen, 1996; Metz *et al.*, 2004). Since the precise nature

and location of the effects of different toxoiding reactions on the PT molecule have not been defined, the PTd present in pertussis vaccines could be modified on either the A-protomer, B-oligomer or both. A possible discrepancy between the *in vivo* and *in vitro* assays for the vaccine products could conceivably occur if the detoxification procedure was modifying mainly the B-oligomer, hence preventing the binding of the holotoxin to receptor sites on the cell surface and entry of the A-protomer into the cells. Furthermore, detoxification might affect parts of the PT molecule involved in binding to ATP, or internalisation and intracellular translocation (Hausman *et al.*, 1990; *el* Baya *et al.*, 1999), either alone or together with the receptor binding, ultimately preventing the entry of the A-protomer into the cytoplasm. Therefore, to understand better the effect of chemical detoxification on these PT subunits, both of the biological functions of PT should be measured.

Pertussis-based combination vaccines have been introduced with the aim of simplifying the immunisation of children against multiple diseases. More antigen components in the combination vaccine formulations could add further complications to the test systems. For native PT, the ADP-ribosylation activity determined by the E-HPLC coupled assay was found to correlate well with the results obtained in HIST (Yuen *et al.*, 2002). However, a correlation between enzyme activity and HIST could not be established for most vaccine formulations assessed in the present study. The HIST is based on the ability of PT to sensitise mice and subsequently cause anaphylactic shock after histamine challenge. As mentioned in Chapter 1, Section 1.4.3.3, the molecular mechanism of this phenomenon is not clear. It cannot be ruled out that potential interaction between residual PT and components or formulation factors in a combination vaccine could also affect the outcome of HIST. Further investigation is needed.

In summary, the ADP-ribosylation assay was further optimised and standardised. Levels of residual PT ADP-ribosylation activity varied between different acellular pertussis-based combination vaccine products. Different detoxification procedures as well as formulation factors could contribute to these variations. A level of enzymatic activity that would be significant in relation to the reactivity seen in the HIST could not be defined. Relying solely on the residual PT enzyme activity in vaccines may not fully reflect the *in vivo* reactivity observed by the HIST. Refinement of the *in vitro* test to include a step which monitors the B-subunit activity of PT may provide a better correlation with the *in vivo* HIST.

4.2 Development of a carbohydrate-binding assay for pertussis toxin and toxoid

Both A- (S1) and B- (S2-S5) subunits functions are involved in histamine sensitisation, where the carbohydrate binding subunits S2-S5 are involved in the binding to host cell receptors, followed by entry of the toxin into the target cells and the subsequent ADP-ribosylation of G proteins by the S1 subunit. Therefore, as suggested in the previous section, a better correlation may be achieved by taking account of both the enzymatic and binding activities of PT when comparing activity in the HIST. In this study a binding assay was developed based on the carbohydrate-binding properties of PT.

4.2.1 Carbohydrate-binding specificity of PT

It is well documented that PT is a carbohydrate binding protein but the majority of the methods used to characterise the carbohydrate binding specificities have been indirect: sequential degradation followed by reglycosylation, deglycosylation by chemical or enzymatic approaches (Armstrong *et al.*, 1988; Hausman *et al.*, 1993; Menozzi *et al.*,

2002) using cell lines with glycosylation defects, inhibition of binding to model receptors or biological activities with glycoproteins or high concentrations of monosaccharides (Sekura and Zhang, 1985; Brennan *et al.*, 1988; Witvliet *et al.*, 1989; Tyrell *et al.*, 1989; Heerze and Armstrong, 1990; van't Wout *et al.*, 1992).

In the present study, using an ELISA system and biotinylated oligosaccharides with defined structures, the precise structural carbohydrate binding specificities of PT were evaluated quantitatively. The results showed that PT had a high binding preference for multiantennary *N*-glycans such as NA3, NA4, A3 and A4, with the highest affinity towards the fully sialylated structures. Preliminary studies indicated that PT was able to bind better to *N*-acetyl (A3) than to the *N*-glycolyl form of sialic acid (A3-1). Binding activity was also found to be adversely affected by core fucosylation of A3 and A4. The high bindings observed for A3 were in agreement with the binding activities observed to the glycoprotein fetuin, which consists predominantly of A3. hAGP also contains sialylated tri- and tetra-antennary type *N*-glycans (Fournet *et al.*, 1978; Yoshima *et al.*, 1981) but, in the present assay system, higher coating concentrations (>8 μg/well) were required to achieve similar binding to that obtained with fetuin at 1 μg/well. The differences in binding activities of PT to these two glycoproteins may be due to different *N*-glycan presentation or because of possible inferior coating of hAGP to the ELISA plates due to its higher carbohydrate content (45%). To my knowledge, this is the first report demonstrating the preferential binding of PT to fully sialylated tri- and tetra-antennary *N*-glycan structures. The method described herein avoids the interpretational problems of carbohydrate structural heterogeneity seen in glycoproteins-based experiments. It also allows for the direct comparison of PT-binding specificities and has the potential ability to differentiate binding activities to different structures on glycoprotein ligands.

Previously, the binding of PT to a fetuin-coated plate was inhibited by sialyllactose (Witvliet *et al.*, 1989). However, in the present study, little or no binding was found to siayllactose. The difference between those data and the data reported here could be due to the use of higher concentration of trisaccharide (12.5mM, Witvliet *et al.*, 1989). The binding of PT to fetuin could also be inhibited by glycoproteins carrying biantennary structures (Sekura and Zhang, 1985; Heerze and Armstrong, 1990; Heerze *et al.*, 1991). In the present study, there was negligible binding of PT to biantennary *N*-glycan structures (A2) presenting either as glycoprotein (hTf, bAGP, 1 µg coating) or *N*-glycan ligands, at the concentrations studied. It was interesting that the binding activities for bAGP and hTf at 8 µg/well were 1.167 and 0.841 respectively and unlike the other glycoproteins, the binding activities reduced sharply to near background values at the 4 µg coating level. As mentioned previously the binding of PT to Fet was maximum above 1 µg coating concentration. bTG and hTf have a lower (~8%) carbohydrate content (Spiro and Spiro, 1965; Riebe and Thorn, 1991) than Fet which has a carbohydrate content of approximately 20%. Therefore it is likely that the observed maximum binding to 1 µg Fet is due to optimal presentation of higher amounts of carbohydrate present on fetuin rather than the saturated coating of wells (Spiro *et al.*, 1960). Although bAGP contains approximately 35% carbohydrate by weight, both bAGP and hTf consist predominantly of sialylated biantennary structures (Nakano *et al.*, 2004; Spik *et al.*, 1975) whereas Fet consists predominantly of tri-antennary structures (Takasaki and Kobata, 1986). Therefore, apart from the higher carbohydrate content, the overall charge of the carbohydrate content may also be critical for binding and possibly explain the very low binding observed to A2. This discrepancy in the interaction of PT with biantennary structures may be due to differences between the methods employed in different laboratories. It is likely that the presentation of

biantennary structures for PT binding may be different in the various assay systems. The much lower binding affinity of A2 compared to A3 was corroborated in studies using fetuin (A3) and transferrin (A2) as ligands where fetuin was reported to be approximately 500 times better than transferrin in binding inhibition experiments (Tyrell *et al.*, 1989). Also, in a study by Heerze and Armstrong (1990), inhibition of PT binding to fetuin was approximately 150 times higher with human fibrinogen than with human transferrin, although both glycoproteins carry identical sialyl α2,6 biantennary structures (Spik *et al.*, 1975; Townsend *et al.*, 1982). Although the weakly-binding disialylated biantennary *N*-glycans (A2), as observed in this study, have been used successfully to identify carbohydrate binding sites on PT S2 and S3 subunits by X-ray crystallography (Stein *et al.*, 1994b), it would be interesting to determine if the stronger binding tri- or tetra-sialylated (A3, A4) and even the neutral multiantennary *N*-glycans (NA3, NA4) would react to the same binding sites, since regions other than those identified in that study are also implicated in receptor binding (Saukkonen *et al.*, 1992; van't Wout *et al.*, 1992; Lobet *et al.*, 1993; Loosmore *et al.*, 1993; Stein *et al.*, 1994). These regions mainly lie in the N-terminal domains of S2 and S3 that resembles mammalian C-type lectins and, in one study, a free peptide spanning the amino acid sequence of the S3 subunit of pertussis toxin (44-58) was found to bind to fetuin and α_1-acid glycoprotein (Tallet *et al.*, 1993).

4.2.2 Binding of PT to glycolipids

PT was shown to bind to GT1b and GD1a glycolipids using a dot blot method. Consistent with these findings, PT bound to mixed brain ganglioside (Tallet *et al.*, 1993) and to GD1a (Hausman *et al.*, 1993). Mixed brain ganglioside contains approximately 18% GM1, 55% GD1a, 15% GD1b, 10% GT1b and 2% other

gangliosides (http://www.merckbiosciences.co.uk/html/CBC/home.html; for structures see Chapter 2, Table 2.2). Although binding was not observed to all the neoglycolipids or glycolipids (Chapter 2, Table 2.2) tested using microwell binding assays, it is probably due to inadequate coating since it has been reported that approximately 15% of the acidic, 40% of the neutral glycolipids and 20% of neoglycolipids remains associated within microwells after washing procedures (Green *et al.*, 1992; Bierfreund *et al.*, 1998). Fukui *et al*, (2002) reported that 50% of acidic and neutral neoglycolipids were retained when they were immobilised on nitrocellulose and this may explain the weak binding to GT1b and GD1a using dot-blotting. During dot-blotting glycoproteins were loaded onto nitrocellulose membrane using a pipette. It was observed that following application of the sample, the sample spread outwards rapidly. Clustered presentation of oligosacchardies of neoglycolipids is reported to be important requirement for interactions with carbohydrate-binding proteins (Feizi *et al.*, 1994). Therefore further studies using neoglycolipids applied to nitrocellulose using a microinjector (Fukui *et al.*, 2002) for a concentrated application may provide insights into structural requirements of PT for oligosaccharides structures found on glycolipids.

4.2.3 Comparison of PT and PTd binding in glycoconjugate-capture ELISA

PT in its detoxified form (PTd) is an important antigen present in all ACVs. It is usually inactivated by chemical treatment with formaldehyde, glutaraldehyde, or both, or hydrogen peroxide or tetranitromethane (Siber *et al.*, 1991; Edwards *et al.*, 1995). These different detoxification procedures result in different amino acid side-chain modifications and changes in conformational and epitope binding patterns for the resulting PTds (Burns *et al.*, 1987; Ibsen, 1996). Various attempts have been made by several workers to identify the binding sites of PT or to explore mechanisms of

detoxification. Subunits S2 and S3 were found to be involved in binding to fetuin since construction of *B. pertussis* strains in which the S2 or S3 gene was deleted and substituted with S3 or S2 respectively resulted in production of mutant PTs containing two copies of S3 or S2 respectively, both with lower binding activities (Raze *et al.*, 1998). However, the extent of binding to specific oligosaccharide structure(s) mediated by S2 and S3 is unclear (Lang *et al.*, 1989; Schmidt *et al.*, 1989; Witvliet *et al.*, 1989; Loosmore *et al.*, 1993; Raze *et al.*, 1998). One of the commonest chemical detoxification processes uses cross-linking agents formaldehyde and glutaraldehyde. In the present study, a sample of PTd inactivated by glutaraldehyde was used as an example, for the comparison to PT with respect to their binding activities to *N*-glycans (A3 and NA3).

PTd showed much less binding to Fet, A3 and NA3 in comparison to PT. The extremely low binding activities of PTd to glycoconjugate ligands is not surprising since aldehyde treatments has been reported to modify mainly the B-oligomer as evidenced by the formation of higher molecular weight species in the B-oligomer on SDS-PAGE gels. Changes in the structural conformation of the B-oligomer would be expected to lead to reduced binding (Nogimori *et al.*, 1986; Nencioni *et al.*, 1991; Ibsen, 1996; Fowler *et al.*, 2003). However, the reduction in binding activity of PTd to NA3 was less dramatic than that to A3, where the binding was minimal. This suggests that the toxoiding process for this product mainly alters the binding sites involving sialylated structures rather than sites that bind non-sialylated structures. Such a differential modification of the subunits with chemical detoxification of PT using formaldehyde and pyridine-borane was demonstrated in a previous study by Nogimori *et al.* (1986). The authors found that this modification of PT by reductive methylation did not affect S2-S4 mediated haemagglutination. In contrast, reductive methylation

markedly impaired S3-S4 mediated mitogenicity. The present study provides the first evidence that glutaraldehyde detoxification causes the loss of PT binding ability to sialylated multiantennary structures but the ability to bind neutral multiantennary structures is partially retained. To date, the mechanisms and sites of chemical detoxification of the PT molecule remain unclear (Nogimori *et al.*, 1984; Nicosia *et al.*, 1986; Gupta *et al.*, 1991; Nencioni *et al.*, 1991; Petre *et al.*, 1996). Glutaraldehyde or formaldehyde treatment of PT have differential effects on its biological activities (Gupta *et al.*, 1991) and other factors during the detoxification process itself such as, reactant concentrations, matrix, and the availability of the reactive amino acid in the protein solution, could also affect the extent of chemical modification. The defined oligosaccharide-capture ELISA developed in the present study would be useful for determining the specificity and location of the carbohydrate binding sites in the different subunits/parts of the PT molecule, and, furthermore, it could be useful for investigating the mechanisms and sites of action of different chemical treatments on the carbohydrate-binding properties of PT.

4.2.4 Validation of carbohydrate-binding assay using A3-capture ELISA

Since Fet, A3 and NA3 were found to be suitable ligands for PT binding in the ELISA system, further validation of the binding assay was carried out using A3 coating. PT showed high affinity to this ligand and use of oligosaccharides avoids the interpretational problems of carbohydrate structural heterogeneity seen in glycoprotein-based experiments (Section 4.2.1). In addition, use of the sialylated ligand permitted discrimination between PT and PTd. In combination vaccines, more antigen components could potentially complicate the test system. Investigation of the effect of other antigen components on the binding assay method showed that this method was

specific for PT. This was further supported by the relatively higher binding activities observed for the ACV (DTaP-I) compared with the other ACVs such as DTaP-A. DTaP-I consisted of the genetically-detoxified PT-g, contained about five times less PT antigen, and was stabilised/detoxified with a lower concentration of formaldehyde than DTaP-A. Therefore this assay, in theory, could estimate the integrity of the B-oligomer. A preliminary study on the feasibility of the newly-developed binding assay for application to different types of pertussis-based combination vaccines showed that the assay was reproducible and sensitive, could detect 2 ng PT (~0.21 IU), and hence substantiated its potential for monitoring the binding activities of PT/d in vaccine formulations. The results also demonstrated that the binding activities of PT/d recovered from different types of vaccines were variable (Chapter 3, Table 3.3) and it is unlikely that it will be possible to set a common baseline for all products. Furthermore, since different types of vaccines will differ in their detoxification methods and formulation, e.g. antigen concentration, composition and adjuvant types, the influence of these factors on the assay system cannot be completely excluded. For the purpose of monitoring product consistency, it would be reasonable to set up product-dependent specifications. The desorption procedure prior to the binding assay for each type of product could be another source of variation. To avoid this, ideally, one reference batch from each type of vaccine should be included in each assay and would be treated in exactly the same way as the test samples. Despite the above factors and desorption differences between products, comparisons were made in this study using 90/518 as a common reference for DTaP-C and DTaP-D products by using the E-HPLC to calculate total PT binding activity (Chapter 3, Section 3.3.3). However, this is time consuming and needs additional resource to be able to carry out the experiment at the same time. Therefore it may not be practicable.

In summary, using defined oligosaccharide A3, a robust, sensitive binding assay that can be used for investigating the mechanisms of detoxification of PT and for the assessment of PT binding activity in vaccine formulations has been developed. This assay could be used to complement the ADP-ribosylation assay to form the basis of a potential alternative assay to the HIST.

4.3 Exploring the potential application of the ADP-ribosylation and carbohydrate binding assays

4.3.1 Differential modification of A- and B-subunit activities of PT after chemical detoxification

In general, detoxification was found to affect both A- and B- subunit activities. Moreover, there was a greater reduction in binding activity in comparison to enzymatic activity after chemical detoxification. Chemical inactivating reagents such as formaldehyde and glutaraldehyde are reported to primarily react with lysine residues (Nogimori *et al*, 1984, 1986). These lysine residues are present only in the B-oligomer but not in the S1 subunit (Nicosia *et al.*, 1986; Locht and Keith, 1986) and so it is not surprising that the detoxification effect was more apparent on the binding activity than the enzymatic activity. Previous studies have reported that, after detoxification with glutaraldehyde, high molecular weight species were observed on SDS-PAGE gels whilst the S1 subunit retained its original size (Nicosia *et al.*, 1986). Despite the fact that S1 subunit lacks lysine residues, this study and others have shown that both S1 subunit enzymatic and monoclonal antibody binding affinity were reduced after chemical treatment (Burns *et al.*, 1987; Ibsen, 1996; Fowler *et al.*, 2003a, b). It could be argued that the reduction in activity might be due to the constraints imposed by the

extensive cross linking of B-oligomer thereby preventing the accessibility of S1 to its substrate or antibody, rather than chemical modification of the S1 subunit itself. However the intensity of the S1 subunit band decreased on SDS-PAGE gels after detoxification with formaldehyde indicating some modification of the subunit (Nencioni *et al.*, 1991; Petre *et al.*, 1996). Although direct evidence of changes in the A-subunit remain elusive, this is still possible, since both glutaraldehyde and formaldehyde can react with amino acids other than lysine (Habeeb and Hiramoto, 1968; Tome *et al.*, 1985; McIntosh, 1992; Metz *et al.*, 2004).

4.3.2 Comparison of different detoxification procedures

In this study, it was observed that DTaP-A products, in which the PT had been inactivated with formaldehyde/glutaraldehyde, showed a greater reduction in enzymatic activity in comparison to DTaP-C products in which the PT had been inactivated using glutaraldehyde alone. In contrast, despite desorption differences, glutaraldehyde-inactivated DTaP-C products showed a greater reduction in binding activities than formaldehyde/glutaraldehyde-treated PT in vaccines. The reason for the differential observation in A- and B-subunit activities is not clear, since the detoxification procedures vary between different manufacturers. However, the developed *in vitro* assays should be useful for distinguishing differences in the detoxification procedure. Further studies could be carried out to investigate the effect of different chemicals on A- and B- subunit activities of PT.

4.3.3 Comparison of different formulations

In the present study, HIST results obtained for DTaP-C and DTaP-D products were particularly noteworthy. DTaP-C was an intermediate bulk to DTaP-D products and the latter products differed in that they were formulated with additional antigen components. Paradoxically, DTaP-D products, but not the corresponding DTaP-C products, showed reactivity in the HIST. Concomitantly, both A (enzymatic) and B (binding) subunit activities were higher in the DTaP-D products in the *in vitro* assays. This strongly indicated that the differences in reactivity observed *in vivo* could be ascribed to changes in the A- and B-subunit activities of PT (Chapter 3, Section 3.3.3). It was also noteworthy that the enhancement in B-subunit activity was greater than for the A-subunit activity, with the exception of the clinical trial lot and its intermediate bulk. This may have been due to the fact that the detoxification process predominantly targets lysine residues present only in the B-subunit (Nogimori *et al.*, 1986; Nicosia *et al.*, 1986). Therefore, if reversion of the toxoiding process did occur in DTaP-D products then it would be reasonable for the changes to be more evident in the B-subunit than the A-subunit activity (Section 4.3.1). Regardless of the observation that both A- and B- subunit activities were greater in DTaP-D products than the corresponding DTaP-C, the significance of this enhancement in terms of the histamine reactivity is presently unknown. It was difficult to establish the proportion of A- and B-subunit activity required for causing *in vivo* toxicity (see Section 4.3.4.2). In addition the influence of the other additional antigens on the outcome of HIST cannot be ruled out, even though the other vaccine antigens administered individually or in combination with DTaP-C did not show any activity in HIST (DK Xing, personal communication).

Preliminary studies indicated that Tween-80, which is used in the DTaP-D formulation, was a contributor to the enhanced ADP-ribosylation activity in DTaP-D products. The reasons for the effects of Tween-80 on PT enzymatic activity in vaccines are not fully understood but Tween-80 may promote de-aggregation of PT molecules. During the detoxification process, PT has a tendency to aggregate (Jiang and Schwenderman, 2000; Kataoka *et al.*, 2002; Fowler *et al.*, 2003b) and the addition of Tween-80 may release PT concealed or trapped in aggregates. However, when comparing the ratio of binding activity to enzymatic activity in DTaP-C and DTaP-D products, the activity was higher in the latter products suggesting that de-aggregation did not take place, but further studies are required to rule this out. The DTaP-C vaccine was a concentrated product and was diluted to the same concentration as the DTaP-D products (neat) prior to testing of the enzymatic and binding activities. Dilution of the toxoid may facilitate the dispersion of PT from the aggregates. It is possible that, in the present study, sufficient time was not allowed after dilution for the de-aggregation process to reach completion since the DTaP-D products used here had been formulated for months or more. However de-aggregation does not explain why such products showed reactivity in the *in vivo* HIST.

An alternative explanation for the Tween-80 effects may be that re-naturation of the protein occurred, i.e. reversion. The effect of Tween-80 was found to be product specific as its addition to DTaP-C intermediate bulks showed a marked enhancement of enzymatic activity but this did not occur with other products tested (Section 3.3.3.1). This suggests that the Tween-80 effects may depend on the types and extent of chemical detoxification used in the production process, but since the chemical detoxification conditions are different between manufacturers, it is unknown at this stage if the effect of detergent on the enzymatic activity is detoxification procedure specific. However,

under the present experimental conditions, addition of Tween-80 to DTaP-C did not noticeably enhance the binding activity or cause any reactivity in HIST. Therefore, the effect of Tween-80 observed in the present investigation may not entirely explain the enhancement in reactivity in HIST, or the increased binding activity. Again it should be stressed that the significance of this enhancement in enzymatic activity in HIST is not known. Other unidentified formulation factors may be contributing to the higher binding and reactivity *in vivo*. Further investigation is needed into other possible factors that might have contributed to the increased reactivity seen for DTaP-D products.

4.3.4 Relationship between A- and B-subunit activities of PT and the *in vivo* HIST

4.3.4.1 Comparison of A- and B-subunit activities in vaccines and in purified PT 90/518 and their relationship with HIST

In the present study, the residual enzymatic activities found per SHD of the vaccine formulations were approximately 18-200 fold higher (taking the lowest and highest values of enzymatic activities) than in 50 ng of purified PT 90/518. The HSD_{50} of this PT 90/518 is within the range of 16.6-50 ng/mouse (Xing *et al.*, 2002). With no detoxification, the expected ratio would be 400-500 times since non-booster vaccines generally contain about 20-25 µg of PTd. However, in the case of the binding activities in these vaccines, the activities were equal to or 3.6-fold higher than that in 50 ng of purified PT 90/518. This differential reduction of the relative enzymatic and binding activity in vaccines to native toxin could be explained by the fact that detoxification occurs predominantly in the B-subunits (Section 4.3.1, Nogimori *et al.*, 1986; Nicosia *et al.*, 1986). The paradox here is that, even with such high A- and B-subunit activities, most of the vaccine lots did not show reactivity in the HIST. It is generally believed

that the mechanism of toxin action involves three stages: binding; internalisation and translocation of the toxin; and the ADP-ribosylation of G proteins. The binding and enzymatic activity is mediated by the B-oligomer and S1 subunit respectively (Sekura and Zhang, 1985; Ui, 1990). However, the relative roles of the A- and B-subunits in the internalisation process are unclear (Kaslow and Burns, 1992; Spangler *et al.*, 1993; Stein *et al.*, 1994b; el Baya *et al.*, 1997; Castro *et al.*, 2001). It is possible that the discrepancy between the reactivity of PT in the vaccines and purified 90/518 occurred because the detoxification procedure affected parts of the A- or B- subunits involved in translocation of S1 subunit, thereby preventing the ADP-ribosylation of G proteins and reactivity in HIST. On the other hand, others have suggested that PT may transverse the membrane directly (Kaslow and Burns, 1992; Spangler *et al.*, 1993; Stein *et al.*, 1994b).

The precise mechanism of histamine sensitisation and thus the endogenous ligands for PT are unknown. Native PT was shown to bind to several oligosaccharide structures (Chapter 3, Section 3.2.2). For T-cell mitogenicity, PT binds to a 43 kDa and 70 kDa receptor. Activation is believed to be due to the divalent binding mediated by S2 and S3. Although these two subunits share more than 70% sequence similarity (Nicosia *et al.*, 1986; Locht and Keith, 1986), differences in carbohydrate binding specificities have been reported (Armstrong *et al.*, 1988; Brennan *et al.*, 1988; Witvliet *et al.*, 1989). A second reason for the discrepancy could be due to the presentation of ligands in *vivo*. Although vaccines showed binding to oligosaccharide A3 after detoxification, it is possible that the vaccine may not have been able to bind to other carbohydrate structures that are also essential for toxicity *in vivo*. As discussed in Section 4.2.3, future studies using the developed assay should explore the possible use of other ligands and also investigate specificity and location of the carbohydrate-binding sites in the different subunits/parts of the PT molecule. Nevertheless, it is possible that the

mechanism of action of vaccine preparations and purified PT preparations in HIST may be different. The possibility of the interaction between purified PT and other unknown components *in vivo* or PT and formulation factors influencing histamine reactivity cannot be ruled out. For example, previous studies have shown that both native PT and LPS can enhance IL-1β production in the hippocampus (Loscher *et al.*, 2000)

4.3.4.2 Role of A- and B- subunit activities in vaccines and the *in vivo* HIST

DTaP-I products displayed high binding activity but did not show toxicity *in vivo* since the enzyme active site had been eliminated by mutation. Further comparisons of A- and B-subunit activities in DTaP-D, DTaP-E and DTaP-F also highlighted the fact that the higher binding activity of DTaP-D products in comparison to DTaP-E and DTaP-F may have contributed to the toxicity observed *in vivo* for this product. The enzymatic activities were similar between the products (Chapter 3, Figure 3.23 and Table 3.5). The differences in binding activity may have been due to differences in the detoxification procedures. Therefore, both A- and B- subunits appear to play a role in the *in vivo* HIST.

Although these comparisons highlighted a possible role for B-subunits in causing toxicity *in vivo*, it is difficult to determine the relative contributions of A-subunit activity and B-subunit activity. In theory, it might be possible to re-assemble the holotoxin with known amounts of A-subunit and B-subunit activities to establish this relationship. However it might be difficult to produce such a modified holotoxin. PT is arranged in a molar ratio of S1: S2: S3: 2xS4: S5 (Tamura *et al.*, 1982). Thus prior to the assembly of B-oligomer with different levels of activities one would have to identify the effective binding activity of each subunit before any modification. Any structural

modification of individual subunits might cause inappropriate holotoxin assembly and this structural modification might lead to reduced or inappropriate toxin activity, i.e. the carbohydrate-binding specificity might change and cause unpredictable activity *in vivo*.

4.3.5 Establishment of a preliminary relationship between enzymatic, carbohydrate-binding and HIST tests of PT toxicity

A mathematical model was derived according to the method described by the Heinemann working group (Solving equations and inequalities, 2002) to explore the relationship between the *in vitro* and *in vivo* assays. Using the data generated from PT preparation 90/518, the experimental data fitted well with the equation (Section 3.3.4.2). Therefore it seemed reasonable to further apply this model to vaccines. Three death threshold equation lines were generated for DTaP-A, DTaP-C and DTaP-D products, respectively. The need for product-dependent specification was based on the differences in production processes with regard to methods of purification and detoxification, number and quantity of components, incorporated adjuvants and excipients in different vaccine formulations from different manufacturers (Corbel and Xing, 2004). Moreover the extent of detoxification can depend on the reagent used and factors such as pH, availability of reactive amino acids in the protein in solution, reactant concentrations and the matrix can affect the quality of the resulting toxoid thereby resulting in different levels of residual PT activity both in products from different manufacturers and within products (Sections 4.3.2 and 4.3.3.2; Metz *et al.*, 2004). The influence of these factors on the enzymatic, binding and HIST cannot be completely predicted. Although the model for the vaccines was only very preliminary, a relationship between *in vitro* and *in vivo* assays was observed for all vaccine products when applying the E-HPLC cut-off line. It appears that there is product-specific level of enzymatic activity that is critical

for reactivity in HIST. However this is based on the assumption that the death threshold equation line below the E-HPLC cut-off value does not apply. So far, none of the reactive batches showed any reactivity below the HPLC threshold line. This finding is in line with the fact that the histamine sensitisation is dependent on the expression of PT catalytic subunit activity (Pizza *et al.*, 1986; Nencioni *et al.*, 1986). The binding activity only contributed to the reactivity *in vivo* when the ADP-ribosylation activities were above the E-HPLC cut-off line. To replace the HIST, more work needs to be done to evaluate a larger number of vaccines and accumulate a large amount data for meaningful analysis and to judge the appropriateness of the mathematical model. Nevertheless, the results obtained for vaccine formulations A, C and D (Figures 3.25-3.27) suggested that the developed methods form a good basis for the future development of an alternative assay to the HIST. At current stage, if using the E-HPLC cut-off value for the different vaccine formulations (Figure 3.25-3.27), it may reduce the HIST by approximately 70, 40 and 40 % for DTaP-A, C and D products respectively. The E-HPLC-cut off line was also found to be product-specific and the higher enzymatic cut-off line generally paralleled with the higher reactivity seen *in vivo* (e.g. DTaP-D). However, the clinical significance of the reactivity of vaccines in HIST is not known.

In summary, the developed assay could be useful for measuring product-specific consistency and a preliminary relationship between the developed *in vitro* assay and *in vivo* assay was established. Further studies, evaluating a larger number of vaccines is required for meaningful statistical analysis. The methods developed form a good basis for the future development of an alternative assay to the histamine challenge test.

Chapter 5

Proteomic analysis of PT induced CHO cell clustering

5.1 Introduction

The *in vitro* assays for measuring the A- and B- subunit activities of PT (Chapters 4) forms the basis of a potential replacement test for the *in vivo* safety testing of pertussis-based combination vaccines by the HIST. The developed assays are biochemical assays, measuring the enzymatic and binding activities of PT independently and demonstrating different parts of the mechanisms involved in its toxicity. The precise nature of PT-induced toxicity and the target cells involved in histamine sensitisation are unclear and thus present some challenges in developing a suitable alternative *in vitro* model for measuring active PT that is directly reflective of the toxicity observed by the HIST. The CHO cell clustering assay has been used as an *in vitro* test for determination of residual active PT in vaccines. Despite the disadvantages of this assay e.g. interference by adjuvant and possibility of failing to detect PT activity concealed in aggregates of toxoided PT (Chapter 1, Section 1.6.2), the CHO-cell assay can still be used for determination of residual PT in PTd at the bulk stage. Similar to HIST, the mechanism of the CHO cell clustering is not clear (Chapter 1, Section 1.4.3.4). In order to understand how CHO cells are altered by the toxin and hence to give some leads into developing a mechanistic-based alternative assay for the safety testing of pertussis-based combination vaccines, comparative proteomics was used to study the changes and possible mechanisms of PT-cell interactions.

Proteins are effector molecules controlling the biological processes in an organism. It is at the protein level where the biological processes can be modulated, thus changes for example in cell function due to toxin treatment can be linked at the protein level. In general, DNA codes for RNA which is translated to protein therefore, theoretically, studying the genetic (mRNA levels/transcriptomes) or protein profiles of control and toxin-treated cells should give meaningful insights to the mechanism of toxicity.

Nevertheless, studying the protein profiles is considered to be more direct. A single gene can give rise to different proteins and functional genomics studies have indicated that analysis of mRNA levels does not necessarily correlate with protein expression level nor does it indicate the nature of the functional protein product (Gygi *et al.*, 1999; Anderson and Seilhamer, 1997). In addition, several post-translational modifications, such as glycosylation or phosphorylation can affect proteins following translation but cannot be predetermined at the genetic level yet can alter the cell's functions. Therefore, in this study, the protein response was targeted to gain an understanding of the mechanism of PT-induced toxicity.

'Proteomics' is a high throughput analytical approach for expressed proteins of a cell, tissue, or biological fluid in a given time. The proteome of a biological sample (e.g. cell) is a complex and dynamic entity that changes depending on its physiological state. The core technology of proteomics combines protein separation by two dimensional polyacrylamide gel electrophoresis (2D-PAGE) with mass spectrometry for protein identification. An alternative non-gel based strategy suitable for this purpose involves the use of the isotope coded affinity tag (ICAT) method (reviewed by Geert *et al*, 2005). In this study, the former method was used for investigating differentially-expressed proteins after PT challenge of CHO cells.

2D-PAGE technique sorts proteins in two separate steps according to two independent properties: the first-dimension, isolelectric focusing (IEF) and the second dimension step, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). IEF separates proteins according to their isoelectric points (pI). Proteins are amphoteric molecules that carry a positive, negative, or zero net charge (pI) depending on the pH of their surroundings. For IEF, the protein mixture is loaded onto a carrier ampholyte-

generated pH gradient in polyacrylamide gel, and, under the influence of an electric current, the proteins will initially move towards the electrode with the opposite charge. As each protein migrates, the net charge and the mobility will progressively decrease and it will stop migrating at its pI. If the protein diffuses away from its pI, it will pick up a charge and hence move back to the position where it is neutral. This is the focusing effect of IEF, which concentrates the protein at their pIs. SDS-PAGE separates proteins according to their molecular weights. Proteins to be separated by this method are kept under denaturing conditions to produce a linear polypeptide chain using a combination of SDS and reducing agents such as mercaptoethanol or dithiothreitol. SDS is an anionic detergent that breaks up complex protein structures by binding to proteins specifically in a mass ratio of 1.4 g SDS/g protein. This confers a net negative charge to the polypeptide in proportion to its length. The net charge to mass ratio is constant. Polyacrylamide gel acts as a molecular sieve, formed by polymerisation of acrylamide monomers into long polyacrylamide chains and cross-linking of the chains by inclusion of bis-acrylamide, that restrains larger molecules from migrating as fast as smaller molecules. Proteins of different molecular weight ranges can be separated by using the appropriate % T, which corresponds to the total concentration of acylamide monomers (acylamide and bis-acrylamide). Therefore, in an electric field, the negatively charged denatured protein in a polyacrylamide gel will move to the anode and migration will depend on the molecular weight of the protein.

Sample preparation for complete solubilisation, disaggregation, denaturation, and reduction of proteins is essential for IEF and obtaining a good 2-D gel result. These can have a large effect on the appearance of the final gel and need to be determined for a given sample. Allowing sufficient IEF focusing times (which needs to be empirically determined) to separate the proteins is also important to achieve good separation and

well resolved protein spots. Following the establishment of these conditions for a given sample, this technology allows thousands of different proteins to be separated with high resolution, and information such as pI, apparent molecular weight, and the amount of each protein is obtained. These features, and its relative robustness following introduction of immobilised pH gradient (IPG) strips, which are created by covalently incorporating a gradient of acidic and basic buffering groups into a polyacrylamide gel at the time it is cast, has made this separation technology a powerful and popular tool for the analysis of complex protein mixtures (Gorg *et al.*, 2000). 2D-PAGE, however, can be very challenging in order to resolve reproducibly and accurately thousands of separated proteins. The general procedure for performing 2-D PAGE involves: sample preparation; IPG strip rehydration; sample application; IEF; equilibration; SDS-PAGE; staining. Each of these processes needs to be stringently controlled in order to produce gels reproducibly and with high quality. Considering the multi-step procedure, the need for much manual handling and the scale of the proteins involved, reproducibility can be extremely challenging. Sampling solubilisation conditions, sample handling, sample loading, and staining absorption, buffer preparation, gel casting and numerous other processes can affect the resulting gel image and contribute to variability in measured protein separation and intensity. The fact that the result is only known at the end of the experiment makes this method a very long and technically-demanding procedure requiring much skill to master.

Reference proteomic maps of the CHO cell line have been described previously (Champion *et al.*, 1999; Naryzhny *et al.*, 2001; van-Dyk *et al.*, 2003; Hayduk *et al.*, 2004). van-Dyk *et al.* (2003) used the reference map for investigating cellular changes associated with increased production of human growth hormone. In the present study, this technique was applied to examine the interaction of PT with target cells using the

CHO cell line as a model. The effect of PT on the protein profiles of CHO cells has not been reported previously. Changes in protein expression in cells induced by a given toxin should reflect its toxicity. Thus, comparing the protein expression profiles of PT-treated cells against reference non-treated cells may provide an understanding of the mechanism of this assay. Since the dynamic range of proteins depends on the cell status and condition, sampling time is critical. The CHO cell assay is routinely used in our laboratory for monitoring active PT in pertussis vaccine preparations. The sampling time and concentration of PT chosen were known to cause the maximal clustering response.

5.2 Results

5.2.1 Determination of optimal solubilising conditions and IEF times for the proteomic profiling of CHO cells

The protein profiles of CHO cells under three different solubilising conditions were compared by loading equal amounts of protein (100 µg) on each gel, and staining the second-dimensional gel with silver. For optimal sample preparation, three different lysing methods were compared and consisted of: 7 M urea 2 M thiourea lysis buffer; 7 M urea 2 M thiourea lysis buffer with sonication; and 9 M urea only lysis buffer (Chapter 2, Section 2.9.1.3). These methods are commonly used for solubilising various protein samples. Figure 5.1 shows the 2-D gel images using the different lysis methods. The overall protein spot profiles were similar. The use of thiourea in combination with urea as a chaotrope has been reported to improve the solubilisation of hydrophobic membrane proteins and proteins that tend to aggregate (Rapilloud, 1998). Therefore, theoretically this should potentially separate more proteins, and for this reason it was

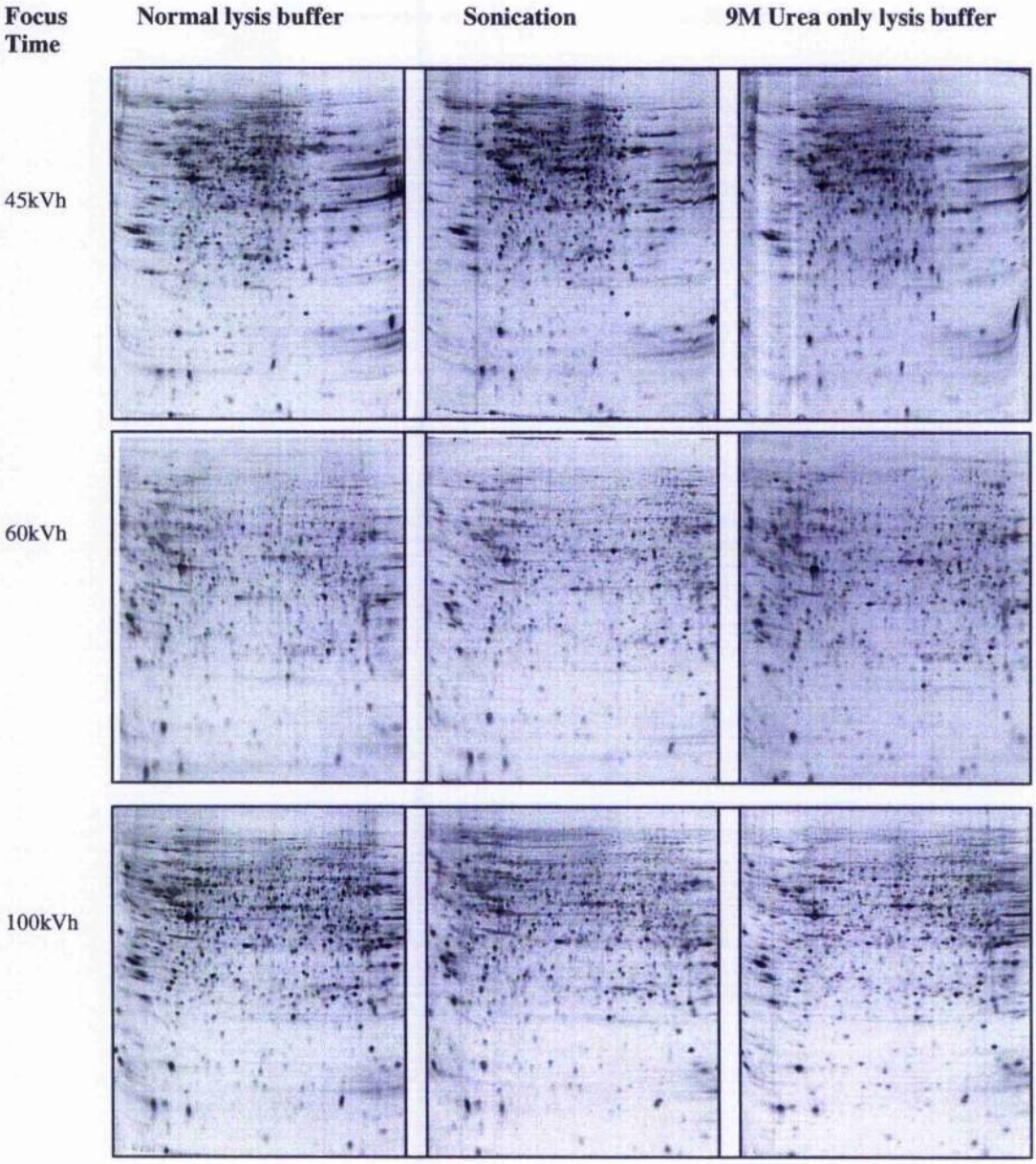


Figure 5.1 The effect of different solubilising methods and IEF times (kVh) on protein separation and resolution in 2D-PAGE. 2D-PAGE was performed as described in Chapter 2, Section 2.10. pH 3-10 NL IPG strips with 100 µg loading of total protein extracts from CHO cells were used for first dimension and second dimension was performed on 12% T polyacrylamide SDS-gel.

chosen for all further experiments. Resolution of the spot separation is also important for high performance 2D-PAGE and can be affected by the IEF times. Three different focusing times were investigated and it was found that the optimum time needed for the IEF pattern to reach the steady state (best resolution) was achieved when using longer focusing times, thus 100 kVh was used for all subsequent experiments (Figure 5.1).

5.2.2 2D-PAGE using narrow range IPG strips

Using a broad-range pH 3-10 IPG strip, a high complexity of protein spots was observed (Figure 5.1) resulting in overcrowding of spots. This could potentially hamper subsequent protein quantitation and identification. To overcome this, overlapping narrow range pH 4-7 and pH 6-9 IPG strips were selected for the purpose of investigating the proteomic profiles of treated CHO cells. Good separation of proteins was achieved under the optimised conditions for IPG strips pH 4-7 (Figure 5.4). On the other hand, under the same conditions with the pH 6-9 IPG strips the basic proteins were not well resolved (Figure 5.2). Shorter focusing times are recommended by the manufacturers for basic proteins and, to improve the separation, different focusing times were investigated. The resulting 2-D electrophoretic separation of basic proteins is shown in Figure 5.2. Under the usual experimental conditions, basic proteins were not well resolved as evidenced by horizontal streaking on the gels. Direct addition of reducing agent DTT at the cathode reportedly improves solubilisation of less soluble proteins (Hoving *et al.*, 2002). However this was not seen in the present study (data not shown). Obtaining good solubilisation for basic proteins is a common problem associated with this technology (Shaw and Riederer, 2003; Henningsen *et al.*, 2002). Nevertheless, several advances in solubilisation methods and strategies for separation of basic proteins such as multiple gel fractionation, organic solvent fractionation and other

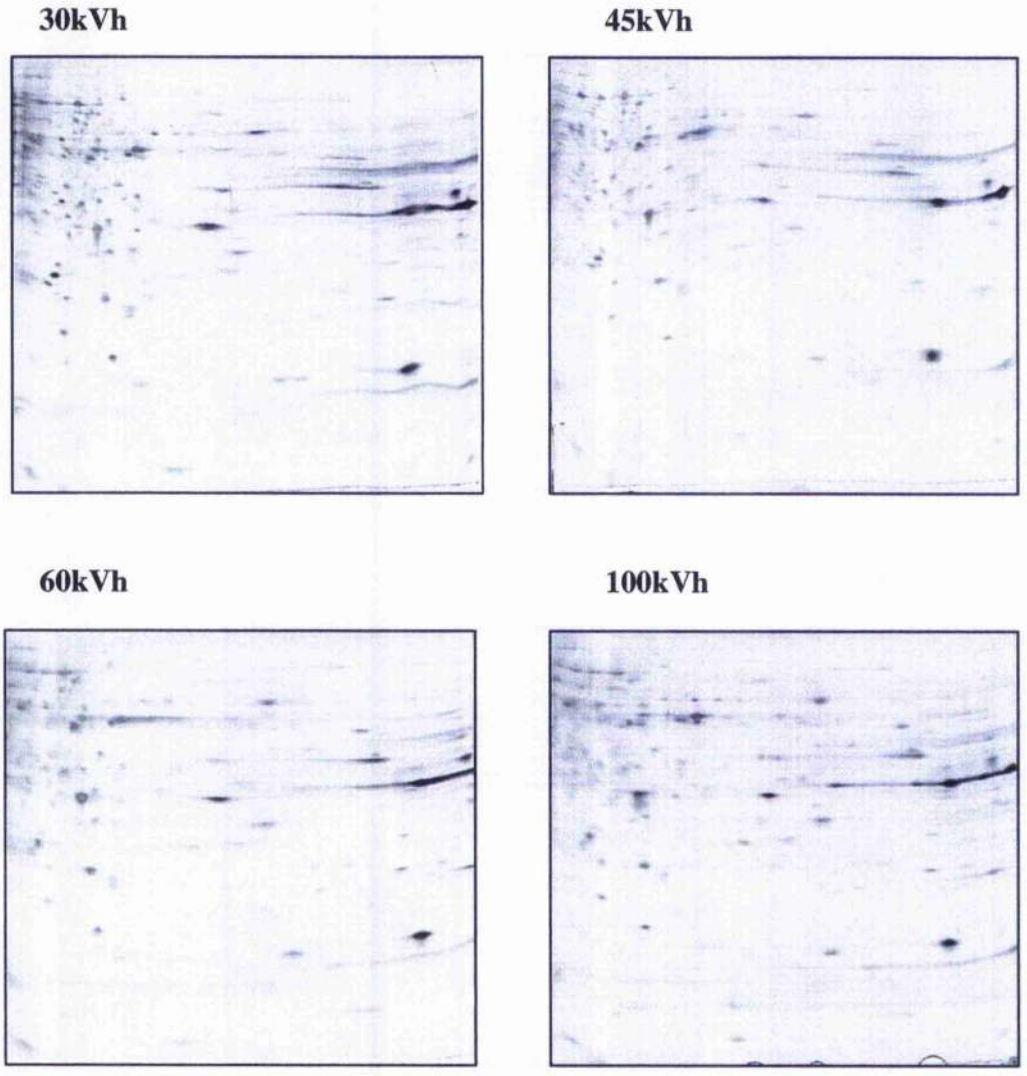


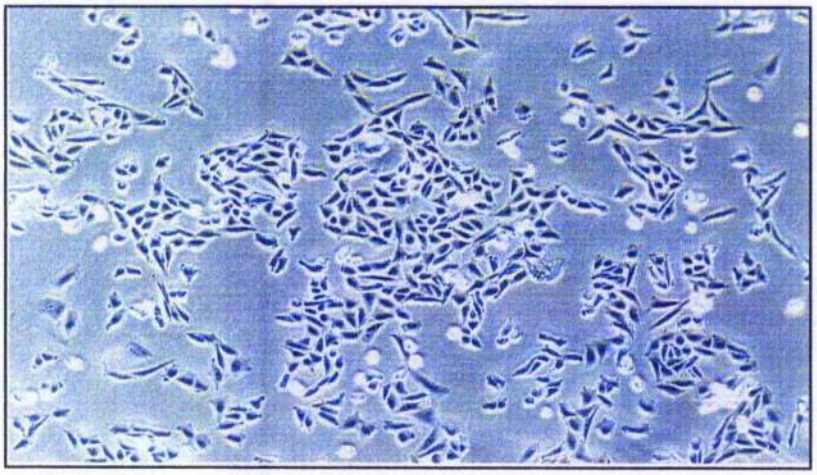
Figure 5.2. The effect of different IEF times (kVh) on protein separation and resolution in 2D-PAGE using pH 6-9 NL IPG strips for first dimension. 100 µg of total protein extracts from CHO cells were used for gel loading and 2D-PAGE was performed as described in Chapter 2, Section 2.10. Second dimension was performed on 12% T polyacrylamide SDS-gel.

fractionation experiments for isolation of integral membrane protein have greatly improved the recovery of these types of proteins (Molloy, 2000). However, due to restricted time, further studies were not carried out. In this study, proteomic analysis of CHO cells was focused in the acidic pH range.

5.2.3 Comparison of control and toxin-treated CHO cell protein expression profiles

PT induces CHO cells to grow in clusters of tightly associated elements (Figure 5.3). At the end of the treatment, the viability of untreated and treated CHO cells, as monitored by trypan blue exclusion, was as high as 95% with the usual experimental conditions. CHO cell lysates from control and PT-treated cells, from three separate experiments were pooled, prior to sample solubilisation. Total cellular proteins (50 µg) were prepared from the control and treated cells and separated using the optimised two-dimensional gel electrophoresis in three replicate gels per treatment. Using the default parameters in the software, more than 1500 spots from each gel were automatically detected. From the three control replicates, control 3 was used as reference image as this gel presented the highest number of well-resolved protein spots (Table 5.1). The spots that matched across 6 gels were individually confirmed manually. All negatively-stained and co-migrated spots were omitted from the analysis as quantitation was no longer accurate. After analysis, 579 spots were presented and matched in all of the 6 gels (Table 5.1). Of those, in comparison to control 3, 262 spots were increased, 220 decreased and 97 remained unchanged in expression level due to PT treatment. All the changes were proportionally quite small and statistical analysis was not feasible because of the low number of gel replicates and intrinsic problems related to this methodology in terms of reproducibility. The gel images of the proteomic profiles of control and treated CHO cells are shown in Figure 5.4.

(a)



(b)

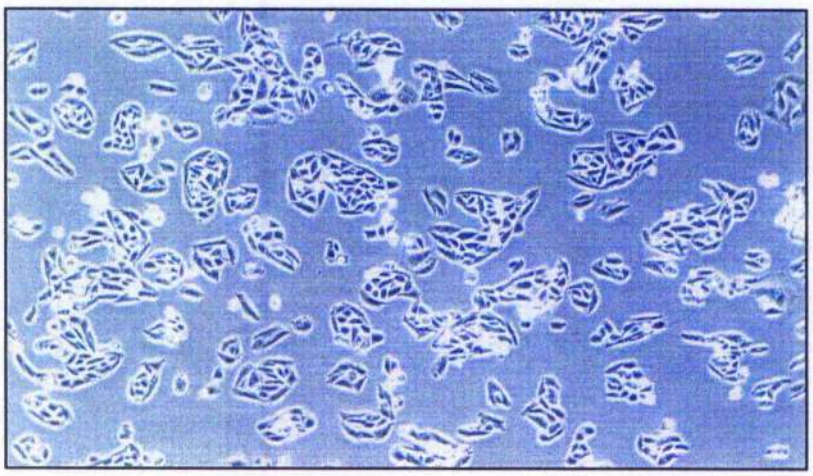


Figure 5.3. PT-induced clustering effect on CHO cells. (a) CHO cells treated with tissue culture medium only for 48 h. (b) CHO cells treated with PT at 64 ng/ml for 48 h. Images were captured using a Nikon Diaphot inverted microscope with phase contrast optics. Magnification 20 x lens. Courtesy of Dr. R. Fleck, NIBSC

Table 5.1. Number of spots identified and matched to reference image.

Gel Name	No. of spots detected	No. of spots matched to reference gel (%)
Control 1	1712	1366 (78)
Control 2	1772	1441 (81)
*Control 3	1782	1782 (100)
Treatment 1	1533	1239 (75)
Treatment 2	1743	1276 (72)
Treatment 3	1741	1304 (74)

ImageMaster 2D Platinum software 5.0 was used for spot analysis and was performed using the default parameters in the software. * reference gel.

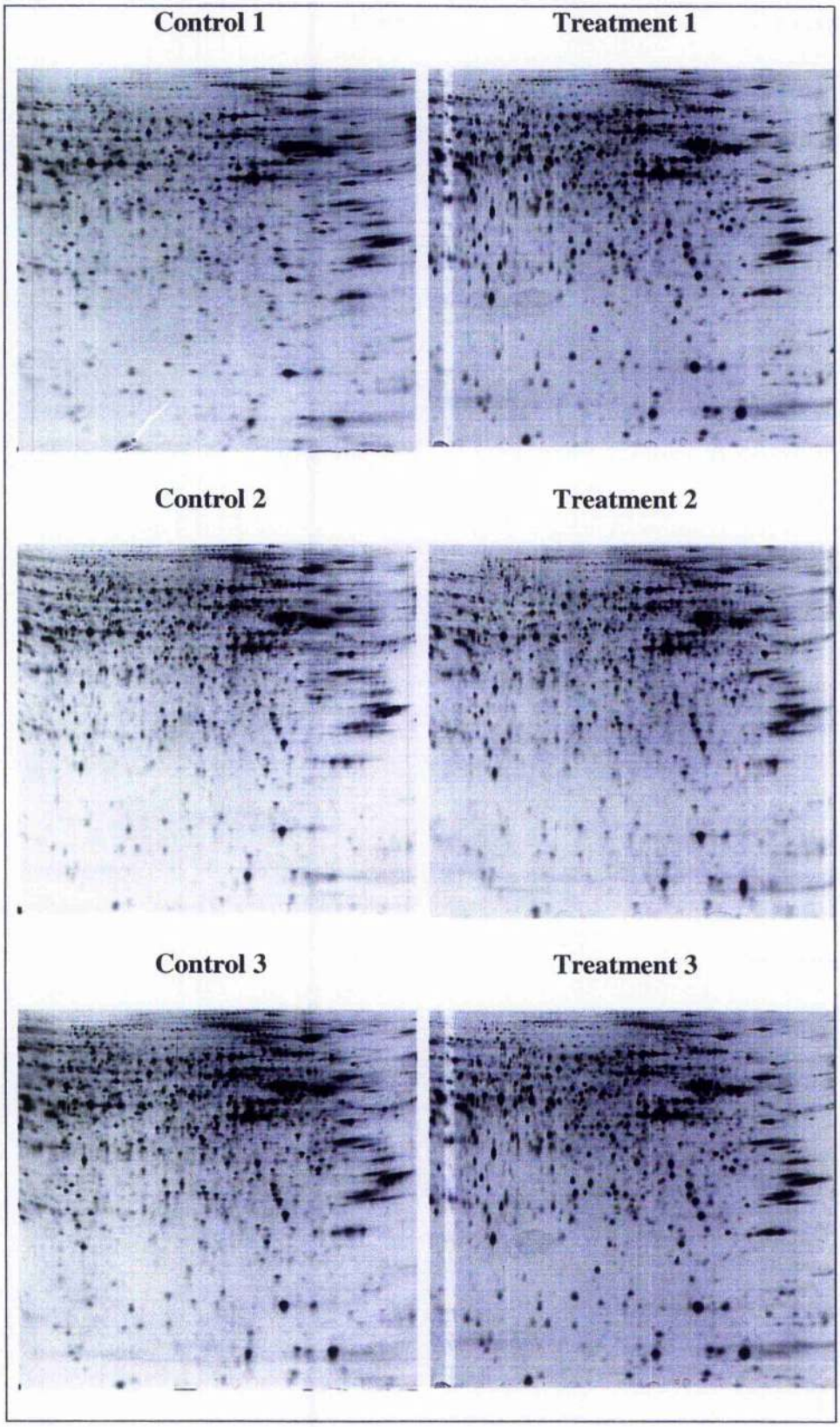


Figure 5.4 2D-PAGE protein profiles of the control and PT-treated CHO cell extracts. 50 µg protein loaded, IEF at pH 4-7, 12% T SDS-PAGE.

5.2.4 Protein Identification

A total of 199 protein spots were excised from each of the two preparative gels and analysed by capillary LC-MS/MS. The CHO genome has not been fully sequenced therefore there is some loss of stringency in assurance of protein identification and for this reason it was preferred to do cross-species sequence homology using UniProt/Swiss-Prot FASTA, which is a curated protein sequence database that provides a high level of annotation, a minimal level of redundancy and a high level of integration with other databases (Apweiler *et al.*, 2004; Bairoch *et al.*, 2005). The MS/MS data were also compared against all entries of the NCBI non-redundant protein database (downloaded 16 May 2005) which contains the translation of all coding sequences presented in the NCBI nucleotide sequence databases. Many of the identified proteins were similar in protein identity regardless of the database used. However, 3 additional proteins were identified, namely vimentin, hypothetical protein 1-2 and cytochrome c oxidase polypeptide Va, when using the NCBI database. The latter protein decreased (1.1-fold) in quantity following PT treatment and the former two proteins were not quantitative. Using UniProt/Swiss-Prot FASTA, a total of 51 protein spots were identified (Table 5.2, Appendix E.3). The 2-D PAGE map of the identified proteins is shown in Figure 5.5. Of the 51 proteins identified, 25 proteins matched to those spots with accurate quantitation. Of these, 88% were found to be differentially expressed in PT-treated cells. 19 proteins increased and 3 proteins decreased in expression. The identified proteins with accurate quantitation were sorted based on their known functions: cell stress associated (5 spots), cell cycle (3 spots), cell signaling (3 spots), protein folding (6 spots) protein degradation (4 spots), protein synthesis (2 spots), membrane and cytoskeletal associated (1 spot) and metabolic enzymes (4 spots) and are shown in Table 5.3.

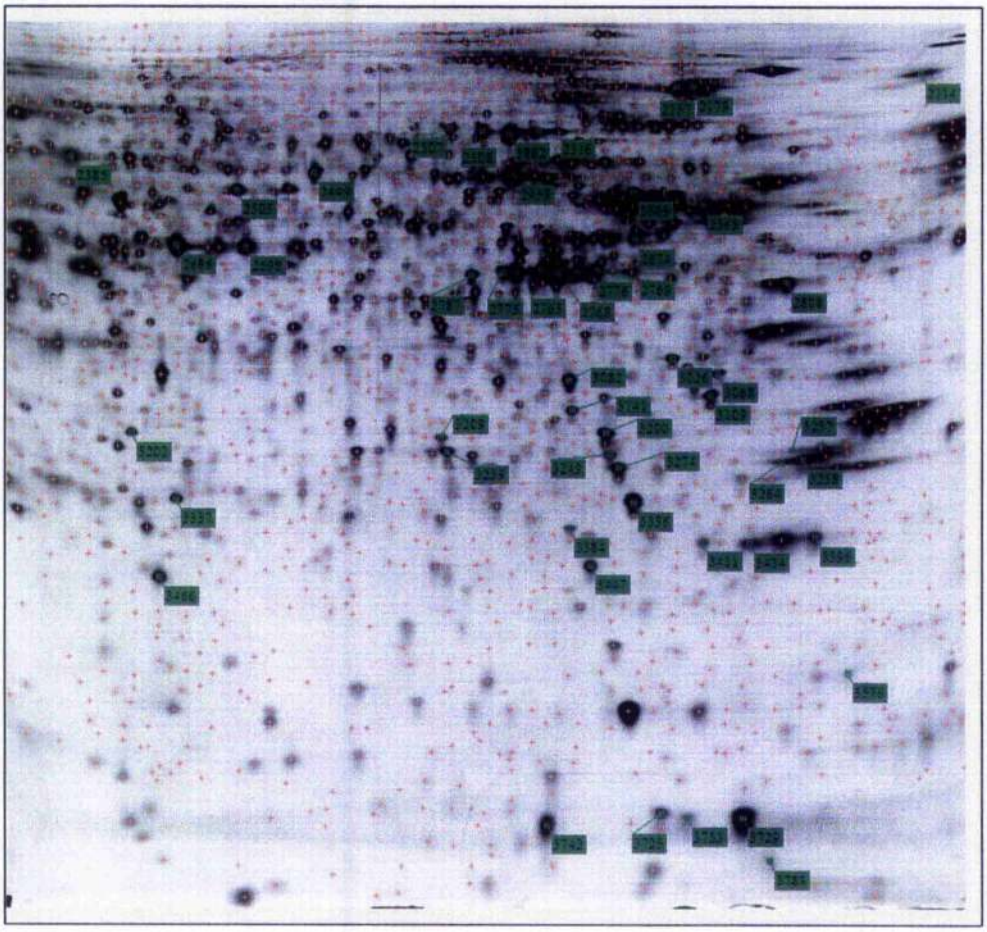


Figure 5.5 2D-PAGE proteome map of CHO cell proteins. 400 µg protein was loaded and the gel developed with MS compatible silver staining.

Table 5.2 Proteins identified in CHO-K1 2D-gels. Proteins that have matched to those with accurate quantitation data showing in red (increased due to PT), blue (decreased due to PT) and green (no change). Black, not quantitative.

Spot ID No	Protein identity and/or homologue to other species	Spot ID No	Protein identity and/or homologue to other species
2114	Calreticulin, *Cricetulus griseus* (Q8K3H7)	2698	Alpha enolase, *Homo sapiens* (P06733)
2157	78 kDa glucose regulated protein, *Homo sapiens* (P11021)	2763	Actin, cytoplasmic 1, *Cricetulus griseus* (sp P48975)
2179	78 kDa glucose regulated protein, *Homo sapiens* (P11021)	2768	Actin, cytoplasmic 1, *Cricetulus griseus* (P48975)
2307	Stress-70 protein, *Cricetulus griseus* (O35501)	2769	Actin, cytoplasmic 1, *Cricetulus griseus* (P48975)
2308	Heat shock cognate 71 kDa protein, *Cricetulus griseus* (P19378)	2775	Actin, aortic smooth muscle, *Homo sapiens* (P62736)
2316	Heat shock cognate 71 kDa protein, *Cricetulus griseus* (P19378)	2776	Actin, cytoplasmic 1, *Cricetulus griseus* (P48975)
2385	T-complex protein 1 subunit beta, *Homo sapiens* (P78371)	2787	Nuclear migration protein nudC (nuclear distribution protein C homolog), *Rattus norvegicus* (Q63525)
2449	T-complex protein 1 subunit alpha, *Cricetulus griseus* (P18279)	2808	40S ribosomal protein SA (p40) (34/67 kDa laminin receptor), *Cricetulus griseus* (P38982)
2459	60 kDa heat shock protein, *Homo sapiens* (P10809)	3026	Elongation factor 1-delta, *Mus musculus* (P57776)
2505	Protein disulphide isomerase A3, *Homo sapiens* (P30101)	3068	Elongation factor 1-delta, *Oryctolagus cuniculus* (P53787)
2509	Vimentin, *Cricetulus griseus* (sp P48670)	3082	Inorganic pyrophosphatase, *Homo sapiens* (Q15181)
2563	Tubulin beta-1 chain, *Cricetulus griseus* (P69893)	3109	Annexin A5 (lipocorin V), *Homo sapiens* (P08758)
2674	Protein disulfide isomerase A6, *Rattus norvegicus* (Q63081)	3141	Microtubule associated protein, RP/EB family, member 1, *MARE1-Homo sapiens* (Q15691)
2686	Alpha enolase, *Homo sapiens* (P06733)		

Table 5.2 continued

Spot ID No	Protein identity and/or homologue to other species	Spot ID No	Protein identity and/or homologue to other species
3200	EF hand domain containing 2, *Homo sapiens* (Q96C19)	3384	Ubiquitin conjugating enzyme E2-25 kDa, *Homo sapiens* (P61086)
	Chloride intracellular channel 1, *Homo sapiens* (O00299)	3396	Translationally controlled tumour protein, *Homo sapiens* (P13693)
3202	Proteasome subunit alpha type 1, *Homo sapiens* (P25786)	3411	Lactoylglutathionelyase, *Rattus norvegicus* (Q6P7Q4)
3209	Proteasome activator complex subunit 2, *Sus scrofa* (Q863Z0)	3414	Proteasome subunit beta type 6, *Rattus norvegicus* (P28073)
3237	14-3-3 protein gamma, (protein kinase C inhibitor protein 1), *Homo sapiens* (P61981)	3447	Peroxiredoxin 2, *Homo sapiens* (P32119)
3239	Prohibitin, *Homo sapiens* (P61086)	3466	Protein DJ-1 oncogene, *Homo sapiens* (Q99497)
3249	Proteasome subunit, alpha type, 3, *Homo sapiens* (P25788)	3576	Myosin regulatory light chain 2, *Homo sapiens* (P19105)
3258	14-3-3 protein beta/alpha (protein kinase C inhibitor protein 1), *Homo sapiens* (P31946)	3725	SH3 domain binding glutamic acid rich like protein, *Homo sapiens* (O75368)
3264	14-3-3 protein beta/alpha (protein kinase C inhibitor protein 1), *Homo sapiens* (P31946)	3729	Thioredoxin, *Rattus norvegicus* (P11232)
3274	Ran specific GTPase activating protein, *Homo sapiens* (P43487)	3742	Galectin-1, *Cricetulus griseus (sp P48538)*
3336	Rho GDP dissociation inhibitor 1, *Homo sapiens* (P52565)	3789	Nuclear transport factor 2, *Homo sapiens* (P61970)
3337	Peroxiredoxin 4, *Homo sapiens* (Q13162)	3862	Heat shock cognate 71 kDa protein, *Cricetulus griseus* (P19378)

Table 5.3 Functions of proteins identified from CHO cells treated with PT.

Function	Protein name (fold change)	Fold change	Reference	Spot ID No.
	up regulated			
Cell stress associated	Thioredoxin	1.8	Amer and Holgren., 2000; Okuyama *et al.*, 2003	3729
	Protein DJ-1	1.3	Taira *et al.*, 2004	3466
	Peroxiredoxin 2	1.2	Chevallet *et al.*, 2003; Choi *et al.*, 2005	3447
	Peroxiredoxin 4	1.2		3337
	Prohibitin	1.2	Winston *et al.*, 2001; Coattes *et al.*, 2001	3239
Cell cycle	Microtubule associated protein RP/EB family member 1	1.2	Morrison *et al.*, 1998; Rogers *et al.*, 2002; Green *et al.*, 2005;	3141
	Ran-specific GTPase activating protein	1.1	Bischoff *et al.*, 1995	3274
	Nuclear migration protein nudC	1.1	Chiu *et al.*, 1997;	2787
Protein degradation	Proteasome activator complex subunit 2	1.5	Ciechanover., 1998; Schwarz *et al.*, 2000	3209
	Proteosome subunit alpha type 1	1.2	Ciechanover., 1998	3202
	Proteosome subunit alpha type 3	1.2	Ciechanover., 1998	3249
	Ubiquitin-conjugating enzyme E2-25 kDa	1.1	Ciechanover., 1998; Kalchman *et al.*, 1996	3384
Protein folding	Protein disulphide-isomerase A3 (Erp57)	1.3	Frickel *et al.*, 2002; Frickel *et al.*, 2004	2505
	Prohibitin	1.2	Nijtmans *et al.*, 2000	3239
	T-complex protein subunit beta	1.2	Llorca *et al.*, 2000	2385
Cell signalling	SH3 domain binding glutamic acid rich like protein	1.2	Egeo *et al.*, 1998	3725
	Ran-specific GTPase activating protein	1.1	Bischoff *et al.*, 1995	3274
Protein synthesis	Elongation factor 1-delta	1.1	Shen *et al.*, 1997	3026
Metabolic enzyme	alpha-enolase	1.2	Ogino *et al.*, 2001	2698
	Inorganic pyrophosphatase	1.1	Islam *et al.*, 2003	3082
Membrane and cytoskeletal related	Annexin A5	1.6	Tzima *et al.*, 1999, 2000; Mollenhauer *et al.*, 1997	3109
	down regulated			
Protein folding	Stress-70 protein	1.2	Singh *et al.*, 1997	2307
	Heat Shock cognate 71 kDa protein	1.1	Ahmad *et al.*, 1990	2308
Metabolic enzyme	alpha-enolase	1.1	Ogino *et al.*, 2001	2686
	no-change			
Protein folding	T complex protein 1 subunit alpha	0	Llorca *et al.*, 2000	2449
Protein synthesis	Elongation factor 1-delta	0	Shen *et al.*, 1997	3068
Cell signalling	Rho GDP dissociation inhibitor 1	0	Leffers et al., 1993	3336
Metabolic enzyme	Lactoylglutathione lysase	0	Chen *et al.*, 2004	3411

5.3 Discussion

PT induces a characteristic clustering of CHO cells and has been used as an *in vitro* test for control of the residual PT in pertussis toxoids at the bulk stage where the PT has not been adsorbed to aluminium salt. The mechanism by which PT induces clustering in this assay is unclear and therefore a comparative proteomic study of this cell clustering effect would help to understand the mechanism and may give some leads into developing a mechanistic-based alternative assay for the safety testing of pertussis-based combination vaccines.

5.3.1 Proteome map of CHO cells

A 2-D proteomic reference map of CHO cell proteins of pH range 4-7, comprising 51 identified proteins has been established. The number of proteins identified was relatively high in comparison to previous studies (Champion *et al.*, 1999; Van Dyk *et al.*, 2003; Lee *et al.*, 2003). However, the number of identified proteins was low in comparison to the total number of protein spots studied (199 protein spots). The apparent low success rate in protein identification in this study is likely to be due to the interference of the silver stain in the ion trap mass spectrometer, the low abundance of some of the proteins and the presence of spots containing multiple proteins (Scheler *et al.*, 1998; Vipond *et al.*, 2006). Using fluorescence staining combined with tandem time-of-flight MS for protein identification, Hayduk *et al.* (2004) provided the most comprehensive CHO 2D map to date, in which 224/274 proteins studied were identified. The CHO cell genome has not been fully sequenced. The combination of reduced sensitivity as a result of using silver staining, thereby reducing the sequence coverage of peptide, followed by cross-species database matching is likely to be a major

contributor to the lower number of proteins identified in this study. Proteomics is often described as the quantitative and qualitative analysis of the expression of all proteins existing within a cell but it is well accepted that this is very difficult to achieve due to technical limitations of the methodology. For example, in this study, it was observed that the gel resolution was compromised when using broad range IPG strip due to overcrowding of proteins. The problem of resolution with the use of 2D gel electrophoresis is also highlighted by fact that the number of spots detected using narrow range (pH 4-7, ~1700) and broad range (pH 3-10, ~1800) IPG strips in IEF, as used by Hayduk *et al.* (2004), were relatively comparable. Considering the high complexity of CHO cell proteins, and the moderate success rate of protein identification, further studies are still required for establishing a good representation of the CHO cell proteome. This could possibly be achieved by using overlapping zoom-in IPG strips with 1 to 1.5 pH units, larger gel formats (24 cm) that reportedly improve sample separation (Gorg *et al.*, 2000; Wildgruber *et al.*, 2000), and the use of fluorescence staining methods or ammoniacal silver staining for increased sensitivity for protein identification (Westermeier and Marouga, 2005; Chevallet *et al.*, 2006). This will allow for an in-depth analysis of the CHO cell proteome.

5.3.2 Possible roles of differentially expressed proteins in CHO cells treated with PT

Previous studies have reported that changes in cell function to a diseased state are not necessarily correlated with gross or dramatic protein changes (Shapiro *et al.*, 2003; Renieri *et al.*, 2003). Therefore it is conceivable that, in this preliminary study, PT-induced clustering of CHO cells involved subtle changes in various cellular functions that are mainly related to cell stress, cell cycle and protein degradation and folding. The

possible implications of the identified proteins in CHO cell toxicity will be discussed hereafter.

At present, it is known that PT-induced clustering of CHO cells is preceded by binding of the toxin to a 165 kDa membrane glycoprotein followed by retrograde transport through endosomes and the Golgi apparatus (Xu and Barbieri, 1995, 1996; el Baya *et al.*, 1997a,b) and possibly the endoplasmic reticulum (ER) (Hazes *et al.*, 1996; Hazes and Read, 1997; Carbonetti *et al.*, 1999; Castro *et al.*, 2001) prior to the final translocation of the S1 across the plasma membrane and the subsequent ADP-ribosylation of G proteins (Xu and Barbieri, 1995). The possible translocation of PT S1 subunit from the ER is of interest since several of the identified proteins indicate routing activities, possibly of PT to ER and the involvement of ER associated degradation (ERAD) pathway for possible retrotranslocation of the catalytic domain. This ERAD pathway, for the delivery of the enzymatic domain to the cytosol has been shown to be exploited by several AB type toxins (Blanke, 2006). The ER of the cell operates a quality control system that identifies misfolded proteins, transports them into the cytoplasm and then targets them for degradation by the proteosome following ubiquitination (Rutishauser and Spiess, 2002; Spear and Ng, 2003). In this study, the observation of increased (1) ubiquitin-conjugating enzyme E2-25 kDa, which is involved in marking proteins for degradation (Kalchman *et al.*, 1996; Kikuchi *et al.*, 2000); (2) increases in proteosomal activity (Ciechanover *et al.*, 1998) (proteosome activator complex subunit 2, proteosome subunit alpha type 1, proteosome subunit alpha type 3); and (3) increase in protein disulphide isomerase (Erp57) which is a component of the calnexin/calreticulin chaperone cycle system (Frickel *et al.*, 2002, 2004), supports the involvement of the ER and ERAD pathway after PT treatment. Interestingly, ricin, an AB type bacterial toxin, uses the ERAD pathway for translocation of the active A

chain into the cytosol of the host cell. This toxin travels from the Golgi to ER by binding via the lectin activity of the ricin B chain to the molecular chaperone protein calreticulin, which contains a terminally galactosylated oligosaccharide (Day *et al.*, 2001). As mentioned above, Erp57 increased after PT treatment. This protein assists in the proper folding of newly synthesised glycoproteins by the formation of disulphide bonds. However, the interaction of this protein with the molecular chaperones calnexin/calreticulin is imperative for its function (Frickel *et al.*, 2002, 2004). Calreticulin was identified from CHO cells but was not quantitative e.g. non reproducible spot pattern between each gel. PT can bind to terminally galactosylated oligosaccharide structures (Chapter 3, Figure 3.9), therefore it is tempting to speculate that PT may also gain access to ER by binding to the carbohydrate moieties of calreticulin.

ADP-ribosylation of intracellular G proteins by PT is required for CHO cell toxicity but the mechanism(s) leading to the clustered morphology is presently unclear. Fluorescence microscopy studies implicated cytoskeletal proteins as cellular targets for PT since a small percentage of actin filaments were remained in clustered cells (Scapigliali *et al.*, 1988). In this study, due to difficulties in quantitation as a result of saturation with silver staining or other problems inherent to the methodology (discussed in Section 5.3.3), the involvement of the identified cytoskeletal proteins (tubulin beta-1 chain, vimentin and 5 protein spots related to actin) in toxin action could not be clarified. However, indirect involvement of cytoskeletal proteins was seen. T-complex protein subunit beta, also known as chaperonin containing TCP-1-beta (CCT), increased following PT treatment. This chaperone protein is known to interact with tubulin and actin, and is required for the final folding of the cytoskeletal proteins to their native structures (Llorca *et al.*, 2000). On the contrary, heat shock cognate 71 kDa protein

(HSP71), which has been suggested to have a role in the *in vivo* assembly and function of microtubules, decreased (Ahmad *et al.*, 1990). These findings suggest that PT may cause alterations to cytoskeletal proteins. Other proteins that were differentially expressed and may also be associated with the cytoskeleton include proteins related to cell cycle: nuclear migration protein nudC; ran-specific GTPase activating protein; and microtubule-associated protein RP/EB family. NudC is necessary for correct formation of mitotic spindles and chromosome separation during mitosis by modulating the dynein/dynactin motor system (Chiu *et al.*, 1997; Sharp *et al.*, 2000). Ran-specific GTPase activating protein, also known as Ran-binding protein 1, is involved in the intracellular cell cycle signalling pathway and has roles in nucleocytoplasmic transport, centrosome cohesion, cell cycle progression, regulating microtubule nucleation, and the organisation and function of the mitototic spindle (Bischoff *et al.*, 1995). Overexpression of Ran-binding protein 1 yields abnormal mitosis (Guarguaglini *et al.*, 2000; Fiore *et al.*, 2003). Microtubule-associated protein RP/EB binds to microtubules and may be involved in spindle function, stabilising microtubules and achoring them at centrosomes (Rogers *et al.*, 2002; Wen *et al*, 2004; Green *et al.*, 2005). PT may be interfering with cell cycle pathways related to the cytoskeletal organisation during cell division. Defects in microtubules and actin molecules during this process may account for the clustered morphology seen in CHO cells and account for the observed changes in chaperone proteins involved in tubulin and actin folding (Yokota *et al.*, 2001). Previous studies have shown that pertussis vaccine caused an increase in surface microvilli, blebs, lamellipodia and filopodia on cultured CHO cells. These change during the cell cycle (Hewlett *et al.*, 1983; Porter *et al.*, 1973).

Annexin is another protein that was differentially up-regulated by PT. Annexins are known to bind phospholipids, actin-based cytoskeletal protein, collagen, and

carbohydrate moieties of sialoglycoproteins and glycosaminoglycans, in a calcium dependent manner. However their exact physiological role is unclear (Mollenhauer *et al.*, 1997; Tzima *et al.*, 1999, 2000; Kirsch *et al.*, 2000). The binding to collagen and glycoconjugates is extracellular whereas its cytoskeletal and membrane functions work intracellularly. Annexin has been suggested to mediate interactions between cytoskeletal proteins and membranes (Tzima *et al.*, 1999; 2000). It is possible that the clustered morphology is due to the interaction of annexin with the actin cytoskeletal proteins. Alternatively annexin may be transported outside the cell and bind to cell surface glycoconjugate receptors causing cell adhesion.

Other cellular functions associated with toxicity are related to cell stress. PT induced an increase in thioredoxin, a small disulphide reducing enzyme that acts as a hydrogen donor (Arner and Holmgren, 2001). This is of particular interest since it is cited in the literature that reduction of the S1 disulphide bond is essential for ADP-ribosyltransferase activity (Kaslow and Burns 1992). However the increase in thioredoxin is more likely to be due to oxidative stress since numerous proteins important in cellular antioxidant defences were increased, including thioredoxin, peroxiredoxin 2, peroxiredoxin 4 and protein DJ1 (Table 5.2). In addition, thioredoxin has been implicated to block oxidative stress induced-apoptosis mediated by serum deprivation and 1-methyl-4-phenylpyridinium via the suppression of cytochrome c release (Andoh *et al*, 2002). Consistent with this, a decrease (1.1-fold) in cytochrome c oxidase polypeptide Va was observed in this study. High accumulation of ROS can be detrimental to the cell, and eventually lead to apoptosis (Andoh *et al.*, 2002). A balance between the oxidant and antioxidant is important for homeostasis since intracellular redox status is involved in various control mechanisms in signal transduction and gene regulation and has been linked to cellular differentiation, immune response, growth

control, cytoskeletal organisation and apoptosis (Jin *et al.*, 1997; Alexandrova *et al.*, 2006). It appears that PT may be interfering with the regulation of intracellular redox in that the anti-oxidant pathway is switched on constitutively. Prohibitin also known for its anti-apoptotic activity and was increased following PT treatment (Fusaro *et al.*, 2003; Tang *et al.*, 2006). Collectively, these findings indicate that PT may be interfering with pathways involved in apoptosis. This may explain why PT is known not be cytotoxic to cultured cells (Castro *et al.*, 2001).

5.3.3 Technical considerations

In this study, twenty-two identified proteins were found to be differentially expressed due to PT treatment. Nineteen proteins were increased and three proteins were decreased in their expression. However, it should be noted that the degree of changes was below the 2-fold threshold that is often regarded as significant. Despite the many advantages of 2D-gel technology, one of the outstanding problems associated with this technology is reproducibility because of its multi-step procedure. This is exemplified by the observation that only approximately 38.6% of protein spots were presented and matched in all 6 gels due to the inability to reproduce spot patterns, to identify individual proteins even in replicate gels of the same sample, and because of missing spot intensities. Although there are many comparative proteomic studies where there are significant changes in protein expression, the protein changes reported in published studies often only take into account of proteins where the change is or above the 2-fold threshold (Naryzhny and Lee, 2001; Seong *et al.*, 2002; Bhat *et al.*, 2005). In these cases gel-to-gel variation is not a primary concern since statistical validation can be achieved by increasing the number of replicates. However, since all changes were subtle (<2-fold) in this study, it was anticipated that increasing the number of gels for analysis

would not compensate for the variability of the methodology and therefore, in this circumstance, it would be reasonable to assume that this approach is unlikely to differentiate protein quantities between control and treated samples with statistical significance. This, however, can be resolved by using two-dimensional difference in-gel electrophoresis (2D DIGE) and will need to be addressed in future studies to confidently distinguish the subtle protein changes (Marouga *et al.*, 2005).

In summary, a proteomic map of CHO cells has been established and a preliminary investigation of the proteomic changes occuring following treatment of CHO cells with PT was performed. It appears that PT induced CHO cell clustering is a complex process involving subtle changes in various cellular functions. Using this technology hypothetical insight into the mechanism of toxicity is described which related mainly to intracellular transport, cell stress and the cell cycle. It is not known how and if these various cellular pathways correlate and contribute to CHO cell toxicity. The information obtained will be useful for future studies into the possible biological mechanism of PT effect on CHO cells. Further studies are required for more confident and comprehensive understanding of PT toxicity.

Appendix A: HIST solutions

A.1 Stock PT 90/518 (10 µg/ml)

PT 90/518	1 ampoule (20 µg)
PBSG (Appendix A.4)	2 ml

Aliquots of 160 µl were prepared and stored at -20^{0}C for up to 6 months.

A.2 Working concentration of PT for HIST

(i) 50 ng/0.5 ml = 150 µl stock PT 90/518 + 14.85 ml PBSG
(ii) 16.7 ng/ 0.5ml = 3.3 ml PT at 50 ng/SHD 90/518 + 6.7 ml PBSG
(iii) 5.6 ng/ 0.5 ml = 1.12 ml PT 90/518 at 50 ng/SHD + 8.88 ml PBSG

A.3 Phosphate-buffered saline (PBS) and PBS with 0.2% gelatin (PBSG)

(i) PBS

NaCl (anhydrous)	10 g
KCl (anhydrous)	0.75 g
$Na_2HPO_4.12.H_20$	1.44 g
KH_2PO_4	0.125 g

Made up to 1 litre in distilled water.

(ii) PBSG

PBS	1 litre
gelatin	2 g

After mixing, solution was sterilised by autoclaving at a minimum of 15 lbs (121^{0}C) and stored at 2-8^{0}C for up to 2 years.

Appendix B. ADP-ribosylation assay: enzyme reaction stock and working reagents

All the following stock reagents and working solutions were stored in an atmosphere of nitrogen at -20^{0}C unless indicated.

B.1 Stock enzyme assay buffer

1 M Tris/HCl, pH 7.6

B.2 Stock ovalbumin (10 mg/ml)

ovalbumin	30 mg

Made up to 3ml with distilled water

B.3 Stock PT 90/518 (20 µg)

PT 90/518	1 ampoule
Ovalbumin	10 ml at 2 mg/ml

After mixing, aliquots of 100 µl (200 ng) were lyophilised and stored at -20^{0}C as stock reference standards. Prior to enzymatic reaction PT was reconstituted with distilled water and equal volumes of adjuvant and PT were mixed to obtain final desired concentration of PT ranging from 0-500 ng/ml and 13 mM of adjuvant.

B.4 Stock in-house positive control vaccine, Biken

Biken NIBSC code 00/486	1 ampoule
Distilled water	2 ml

After reconstitution, Biken was diluted 1/8 in 2 mg/ml ovalbumin solution and 50 µl aliquots were lyophilised

B.5 Stock dithiothreitol (DTT)

200 mM dissolved in 0.5 M Tris/HCl pH 7.6

B.6 Stock β-NAD

3 mM β-NAD dissolved in 0.1 M Tris/HCl, pH 7.6

B.7 Stock ATP

18 mM ATP dissolved in 0.1 M Tris/HCl pH 7.6

B.8 Stock lysophosphatidylcholine (LPC)

10 mg/ml diluted in $CHCl_3$/MeOH (v/v, 9/1)

B.9 Stock phenylmethylsulfonyl fluoride (PMSF)

100 mM in isopropanalol (ISP)

B.10 Preparation of 1ml working solution of PMSF/LPC

(i) Pipette 150 µl of stock LPC into a 2 ml glass vial and blow dry with a stream of N_2 at RT.

(ii) Add the following to the dried LPC

Stock PMSF	200 µl
ISP	200 µl
0.1 M Tris/HCl pH 7.6	600 µl

This PMSF/LPC working solution was sonicated for 1 min in a water bath at RT before use

B.11 Working ADP-ribosylation reagent

This is freshly prepared by mixing equal volumes of PMSF/LPC, β-NAD and ATP (appendix B.6 to B.10) and is sonicated for 1 min in a water bath at room temperature

B.12 Working substrate concentration

400 μM FAC-$(CH_2)_5$-$G_{\alpha i3}$C20 and FAC-$G_{\alpha i3}$C20 was dissolved in DMSO first then 0.2 M Tris/HCl, pH 7.6 (*v/v* 1/1)

The concentration of substrate should be confirmed by UV absorption at 495 nm in 10 mM NH_4OAc, pH 8.5 (Chapter 2, Section 2.4.1)

Appendix C: Quantification of BTN-oligosaccharide solution

C.1 Orcinol-sulphuric acid reagent

This reagent was prepared on ice in a 1 litre Pyrex conical flask

Orcinol	900 mg
Ethanol	375 ml
18M H_2SO_4	50 ml
Distilled water	25 ml

Concentrated H_2SO_4 was diluted with distilled water slowly and then used to dissolve orcinol. This was then diluted with ethanol and stored at 4-6^{0}C.

Appendix D: 2D-PAGE

D.1 Lysis buffers

(i) Normal lysis buffer (7 M urea and 2 M thiourea)

Tris 1.0 M pH 8.0	0.25 ml
Mg acetate 0.5 M	0.25 ml
Urea	10.6 g
Thiourea	3.8 g
CHAPS	1 g

Made up to 25 ml with distilled water

(ii) 9 M urea only lysis buffer

Tris 1.0M pH 8.0	0.25 ml
Mg acetate 0.5 M	0.25 ml
Urea	13.5 g
CHAPS	1 g

Made up to 25 ml with distilled water. 1% of protease inhibitor mix (Amersham) was added to both lysis buffers and was then aliquoted and stored at -20^{0}C for 6 months.

D.2 Rehydration buffer

Urea	10.6 g
Thiourea	3.8 g
CHAPS	1.0 g
Pharmalyte 3-10 (1%)	0.25 ml
DTT (2mg/ml)	50 mg

Made up to 25 ml in distilled water, and a trace of bromophenol blue (100 µl of 1% solution) was added, aliquoted and stored at -20^{0}C

D.3 IEF times

Current (mA) and Power (W):

(i) Total volt hour ~ 30000

Step	Volts	Ma	W	Volt hours	Time
1	500	1	5	1	
2	500	1	5	2500	5 h
3	3500	1	5	22200	6.3 h
4	1000	1	5	5300	5.3 h
5	500	1	5	10000	holding step

(ii) Total volt hour ~45000

Step	Volts	Ma	W	Volt hours	Time
1	500	1	5	1	
2	500	1	5	2500	5 h
3	3500	1	5	40000	11.4 h
4	500	1	5	2500	5 h
5	500	1	5	10000	holding step

(iii) Total volt hour ~60000

Step	Volts	Ma	W	Volt hours	Time
1	500	1	5	1	
2	500	1	5	2500	5 h
3	3500	1	5	46200	13.2 h
4	500	1	5	11300	22.6 h
6	500	1	5	10000	holding step

(iv) Total volt hour ~100000

Step	Volts	Ma	W	Volt hours	Time
1	500	1	5	1	
2	500	1	5	2500	5 h
3	3500	1	5	70000	20 h
4	1000	1	5	15000	15 h
5	500	1	5	12500	25 h
6	500	1	5	10000	holding step

D.4 SDS equilibration buffer

This solution was freshly made

Urea	21 g
Thiourea	7.61 g
1.0 M Tris-HCl pH 6.8	5 ml
Glycerol	15 ml
SDS	1 g

This was made up to 50 ml in distilled water

(i) For equilibration buffer 1, 200 mg DTT was added to 10 ml equilibration buffer
(ii) For equilibration buffer 2, 480 mg iodoacetamide was added to 10 ml equilibration buffer

A trace of bromophenol blue was added to both equilibration buffers.

D.5 Preparation of 12% SDS-PAGE gel using gel caster

Water	81 ml
20% T, 3% C acrylamide/bis	360 ml
1.5 M Tris-HCl, pH 8.8	150 ml

After adding the above components, degas and mix for a minimum of 1 hour then add the following:

10% SDS	6 ml
10% ammonium persulfate (APS)	2.86 ml
TEMED	86 µl

10% SDS and APS was diluted 1.5 M diluted in Tris-HCl, pH 8.8

Gels should be stored at 2-8^{0}C prior to use.

D.6 Gel sealant: 2% molten agarose

Agarose	0.5 g
Running buffer (Appendix C.7)	25 ml
Bromophenol blue	100 µl of 1% solution

Mix and heat mixture

D.7 Running buffer

Glycine	14.3 g
Tris base	2.75 g
SDS 0.93 g	

Made up to 1 litre in distilled water

D.8 Fixing solution

Methanol	100 ml
Acetic acid	25 ml

Made up to 250 ml with distilled water

Appendix E: Experimental data

Appendix E.1 Raw data values for actual deaths (%) observed in HIST using purified PT preparation

PT ng/mouse	16.7	50
n		
1	40	50
2	0	40
3	20	40
4	60	80
5	50	50
6	10	90
7	0	100
8	10	90
9	0	60
10	0	30
11	0	70
12	70	50
13	60	100
14		70
15		80
16		80
17		90
18		60
19		70
20		90
21		90
22		60
23		60
24		80
25		80
26		30
27		40
28		60
29		100
30		100

n, assay number

Appendix E.2: Raw data values used for creating "Death" threshold equation line.

	HIST (% Death)	ADP (E-units)	Binding (potency)
DTaP-A			
Group 1	10	2.08	0.64
	10	2.58	0.53
Group 2	0	1.34	0.53
	0	1.23	0.78
	0	1.37	1
	0	1.41	0.84
	0	0.91	0.93
DTaP-C			
Group 1	0	2.41	0.45
	0	2.37	0.44
Group 2	6.7	4.75	0.52
DTaP-D			
Group 1	25	5.46	0.72
	21	5.5	1
	20	4.03	0.6
	17	4.36	0.56
Group 2	65	5.87	0.77
	40	5.22	0.64
	60	10.15	0.5
	50	6.03	0.38

ADP-ribosylation activity units (E-units) per SHD of test vaccine. For binding, potency values were analysed by parallel line assay using reference vaccine as described in Chapter 2, Section 2.8.9.

Appendix E.3: Proteins identified in CHO-K1 2D-gels

Spot ID number	*File name*	*Protein ID*	*peptide sequence, charge state and amino acid residue position* ** Methionine oxidation*	*Coverage (%)*
3466	130704_data05	**Protein DJ-1 (oncogene DJ1), *PARK7_HUMAN, Q99497***	ALVILAK, 1+, 6 - 12 GAEEMETVIPVDVMR, 2+, 13 - 27	12
3396	130704_data08	**Translationally controlled tumor protein, *TCTP_HUMAN, P13693***	DLISHDEMFSDIYK, 3+, 6 - 19 VKPFMTGAAEQIK, 3+, 111 - 123	16
2808	130704_data13	**40S ribosomal protein SA (p40) (34/67 kDa laminin receptor), *RSSA_CRIGR, P38982***	KSDGIYIINLK, 2+, 41 - 51 SDGIYIINLK, 2+, 42 - 51 LLLAAR, 1+, 57 - 62 AIVAIENPADVSVISSR, 2+, 63 - 79 FAAATGATPIAGR, 2+, 89 - 101 FTPGTFTNQIQAAFR, 2+, 102 - 116 LLVVTDPR, 2+, 120 - 127	24
2563	130704_data16	**Tubulin beta-1 chain, *TBB1_CRIGR, P69893***	IMNTFSVVPSPK, 2+, 163 - 174 LAVNMVPFPR, 2+, 253 - 262 LHFFMPGFAPLTSR, 2+, 263 - 276 ALTVPELTQQVFDAK, 2+, 283 - 297 YLTVAAVFR, 2+, 310 - 318 EVDEQMLNVQNK, 2+, 325 - 336 NSSYFVEWIPNNVK, 2+, 337 - 350 MAVTFIGNSTAIQELFK, 2+, 363 - 379 ISEQFTAMFR, 2+, 381 - 390	30

Proteins identified in CHO-K1 2-D gels cont.

Spot ID number	*File name*	*Protein ID*	*peptide sequence, charge state and amino acid residue position* ** Methionine oxidation*	*Coverage (%)*
2509	130704_data17	**Vimentin, *VIME_CRIGR, P48670***	SLYSSSPGGAYVTR, 2+, 33 - 46 FLEQQNK, 2+, 105 - 111 ILLAELEQLK, 1+ & 2+, 112 - 121 LGDLYEEEMR, 2+, 128 - 137 QVDQLTNDK, 2+, 142 - 150 EEAESTLQSFR, 2+, 179 - 189 KVESLQEEIAFLK, 2+, 205 - 217 FADLSEAANR, 2+, 277 - 286 ETNLESLPLVDTHSK, 2+, 407 - 421 DGQVINETSQHHDDLE, 3+, 433 - 448	26
2449	130704_data19	**T-complex protein 1 subunit alpha, *TCPA_CRIGR, P18279***	SQNVMAAASIANIVK, 2+, 19 - 33 LLEVEHPAAK, 2+, 64 - 73 YPVNSVNILK, 2+, 190 - 199 YFVEAGAMAVR, 2+, 299 - 309 EQLAIAEFAR, 2+, 434 - 443 SLLVIPNTLAVNAAQDSTDLVAK, 2+, 444 - 466 QAGVFEPTIVK, 2+, 500 - 510 FATEAAITILR, 2+, 516 - 526	18
2505	140704_data04	**Protein disulfide isomerase A3, *PDIA3_HUMAN, P30101***	LAPEYEAAATR, 1+ & 2+, 63 - 73 GIVPLAK, 1+, 76 - 82 YGVSGYPTLK, 1+ & 2+, 95 - 104 TADGIVSHLK, 1+, 120 - 129 VMMVAK, 1+, 283 - 288 KFLDAGHK, 1+, 289 - 296 FLDAGHK, 1+, 290 - 296 LNFAVASR, 1+, 297 - 304 FVMQEEFSR, 1+ & 2+, 336 - 344 FLQDYFDGNLK, 1+, 352 - 362 FLQDYFDGNLKR, 2+, 352 - 363 YKELGEK, 1+, 416 - 422 LSKDPNIVIAK, 1+ & 2+, 423 – 433	20

Proteins identified in CHO-K1 2-D gels cont.

Spot ID number	*File name*	*Protein ID*	*peptide sequence, charge state and amino acid residue position* ** Methionine oxidation*	*Coverage (%)*
2698	140704_data05	**Alpha enolase,** ***ENOA_HUMAN, P06733***	AAVPSGASTGIYEALELR, 1+ & 2+, 32 - 49 AVEHINK, 1+, 64 - 70 IGAEVYHNLK, 2+, 183 - 192 DATNVGDEGGFAPNILENK, 2+, 202 - 220 SGKYDLDFK, 1+ & 2+, 253 - 261	15
2686	140704_data06	**Alpha enolase,** ***ENOA_HUMAN, P06733***	AAVPSGASTGIYEALELR, 1+ & 2+, 32 - 49 AVEHINK, 1+, 64 - 70 LMIEMDGTENK, 1+, 92 - 102 GVPLYR, 1+, 126 - 131 IGAEVYHNLK, 1+ & 2+, 183 - 192 DATNVGDEGGFAPNILENK, 2+, 202 - 220 SGKYDLDFK, 1+, 253 - 261 YDLDFKSPDDPSR, 2+, 256 - 268 SPDDPSR, 1+, 262 - 268 YNQLLR, 1+ & 2+, 406 - 411 IEEELGSK , 1+ & 2+, 412 - 419	23
3336	140704_data07	**Rho GDP dissociation inhibitor 1,** ***GDIR_HUMAN, P52565***	SIQEIQELDKDDESLR, 2+, 34 - 49 YKEALLGR, 1+ & 2+, 51 - 58 EALLGR, 1+, 53 - 58 QSFVLK, 1+, 100 - 105 EGVEYR, 1+, 106 - 111 EIVSGMK, 1+, 121 - 127 IDKTDYMVGSYGPR, 1+ & 2+, 139 - 152 FTDDDKTDHLSWEWNLTIK, 2+, 181 - 199	37
3447	140704_data08	**Peroxiredoxin 2,** ***PRDX2_HUMAN, P32119***	KEGGLGPLNIPLLADVTR, 2+ & 3+, 91 - 108 EGGLGPLNIPLLADVTR, 2+, 92 - 108 TDEGIAYR, 1+, 119 - 126 QITVNDLPVGR, 2+, 139 - 149 SVDEALR, 1+, 150 – 156	22

Proteins identified in CHO-K1 2-D gels cont.

Spot ID number	*File name*	*Protein ID*	*peptide sequence, charge state and amino acid residue position* ** Methionine oxidation*	*Coverage (%)*
3200	140704_data09	**EF hand domain containing 2, *EFHD2_HUMAN, Q96C19***	RADLNQGIGEPQSPSR, 2+, 62 - 77 ADLNQGIGEPQSPSR, 2+, 63 - 77 DGFIDLMELK, 1+, 109 - 118 EFLLIFR, 1+, 152 - 158 VQAINVSSR, 1+, 198 - 206 ELQSTFK, 1+, 234 - 240	20
		Chloride intracellular channel protein 1, *CLIC1_HUMAN, O00299*	GVTFNVTTVDTK, 1+, 37 - 48 NSNPALNDNLEK, 1+, 119 - 130 YLSNAYAR, 1+, 208 - 215	13
3082	140704_data10	**Inorganic pyrophosphatase, *IPYR_HUMAN, Q15181***	MEIATK ,1+, 58 - 63 DPLNPIK, 1+, 64 - 70 YVANLFPYK, 1+, 80 - 88 LKPGYLEATVDWFR, 2+, 178 - 191 VPDGKPENEFAFNAEFK, 2+, 195 - 211 WFHHQK, 1+, 283 - 288	20
3729	140704_data12	**Thioredoxin, *THIO_RAT, P11232***	EAFQEALAAAGDK, 1+ & 2+, 8 - 20 VGEFSGANK, 1+, 85 - 93	21
3725	140704_data13	**SH3 domain binding glutamic acid rich like protein, *SH3L1_HUMAN, O75368***	VYIASSSGSTAIK, 1+, 5 - 17 DIAANEENRK, 1+, 38 - 47 ENNAVYAFLGLTAPPGSK, 2+, 87 - 104	36
3742	140704_data17	**Galectin-1, *LEG1_CRIGR, P48538***	SFVLNLGK, 1+, 29 - 36 LPDGHEFK, 1+, 100 - 107	12
3789	190704_data07	**Nuclear transport factor 2, *NTF2_HUMAN, P61970***	LSSLPFQK, 1+, 56 - 63 ADEDPIMGFHQMFLLK, 2+, 91 - 106 LALHNFG, 1+, 121 - 127	24
3576	190704_data15	**Myosin regulatory light chain 2, *MLRM_HUMAN, P19105***	ATSNVFAMFDQSQIQEFK, 2+, 16 - 33 LNGTDPEDVIR, 2+, 92 - 102 FTDEEVDELYR, 2+, 132 - 142 GNFNYIEFTR, 2+, 150 – 159	29

Proteins identified in CHO-K1 2-D gels cont.

Spot ID number	*File name*	*Protein ID*	*peptide sequence, charge state and amino acid residue position* ** Methionine oxidation*	*Coverage (%)*
3414	200704_data03	**Proteasome subunit beta type 6, *PSB6_RAT, P28073***	TTTGSYIANR, 1+ & 2+, 53 - 62 LAAIQQSGVER, 1+ & 2+, 209 - 219 QVLLGDQIPK, 2+, 220 - 229	13
3411	200704_data04	**Lactoylglutathione lyase, *LGUL_RAT, Q6P7Q4***	VLGLTLLQK, 1+, 51 - 59 RFEELGVK, 1+, 140 - 147	9
3384	200704_data06	**Ubiquitin conjugating enzyme E2-25 kDa, *UBC1_HUMAN, P61086***	VDLVDENFTELR, 2+, 28 - 39 YQLEIK, 1+, 55 - 60 IPETYPFNPPK, 2+, 61 - 71 NAVIVALSSK, 1+, 176 - 185	20
3239	200704_data08	**Prohibitin, *PHB_HUMAN, P61086***	VFESIGK, 1+, 5 - 11 FGLALAVAGGVVNSALYNVDAGHR, 2+, 12 - 35 NVPVITGSK, 1+, 75 - 83 DLQNVNITLR, 1+, 84 - 93 ILFRPVASQLPR, 2+, 94 - 105 VLPSITTEILK, 1+ & 2+, 118 - 128 FDAGELITQR, 1+, 134 - 143 QVSDDLTER, 2+, 149 - 157 AATFGLILDDVSLTHLTFGK, 2+, 158 - 177 KLEAAEDIAYQLSR, 2+, 240 - 253	46
3209	200704_data16	**Proteasome activator complex subunit 2, *PSME2_PIG, Q863Z0***	KQVDVFR, 1+, 14 - 20 ETHVMDYR, 1+, 180 - 187	6
3274	200704_data17	**Ran specific GTPase activating protein, *RANG_HUMAN, P43487***	TLEEDEEELFK, 1+ & 2+, 40 - 50 FASENDLPEWK, 2+, 58 - 68 FLNAENAQK, 1+, 142 - 150 VAEKLEALSVK, 1+ \7 2+, 180 - 190 LEALSVK, 1+, 184 – 190	21

Proteins identified in CHO-K1 2-D gels cont.

Spot ID number	*File name*	*Protein ID*	*peptide sequence, charge state and amino acid residue position* ** Methionine oxidation*	*Coverage (%)*
3249	210704_data04	**Proteasome subunit, alpha type, 3,** ***PSA3_HUMAN, P25788***	VFQVEYAMK, 1+, 20 - 28 AVENSSTAIGIR, 1+, 29 - 40 LYEEGSNKR, 1+, 57 - 65 LFNVDR, 1+, 66 - 71 SLADIAR, 1+, 86 - 92 EEASNFR, 1+, 93 - 99 SNFGYNIPLK, 1+ & 2+, 100 - 109 IIYIVHDEVK, 1+, 196 - 205 IIYIVHDEVKDK, 2+, 196 - 207	28
3264	210704_data07	**14-3-3 protein beta/alpha (protein kinase C inhibitor protein 1),** ***1433B_HUMAN, P31946***	LAEQAER, 1+, 13 - 19 AVTEQGHELSNEER, 2+ & 3+, 29 - 42 NLLSVAYK, 1+, 43 - 50 NVVGAR, 1+, 51 - 56 VISSIEQK, 1+, 62 - 69 YLSEVASGDNK, 1+, 129 - 139 QTTVSNSQQAYQEAFEISK, 2+, 140 - 158	30
3237	210704_data08	**14-3-3 protein gamma (protein kinase C inhibitor protein 1),** ***1433G_HUMAN, P61981***	NLLSVAYK, 1+, 42 - 49 NVVGAR, 1+, 50 - 55 VISSIEQK, 1+, 61 - 68 YLAEVATGEK, 1+, 132 - 141 AYSEAHEISK, 1+, 152 - 161	17
3258	210704_data09	**14-3-3 protein beta/alpha (protein kinase C inhibitor protein 1),** ***1433B_HUMAN, P31946***	NLLSVAYK, 1+, 43 - 50 NVVGAR, 1+, 51 - 56 VISSIEQK, 1+, 62 - 69 YLIPNATQPESK, 1+, 105 - 116 YLSEVASGDNK, 1+, 129 - 139 QTTVSNSQQAYQEAFEISK, 2+, 140 - 158	26
3109	210704_data11	**Annexin A5 (lipocorin V),** ***ANXA5_HUMAN, P08758***	VLTEIIASR, 1+, 108 - 116 TPEELR, 1+, 117 - 122 SVSHLR, 1+, 201 - 206 GAGTDDHTLIR, 1+, 260 – 270	10

Proteins identified in CHO-K1 2-D gels cont.

Spot ID number	*File name*	*Protein ID*	*peptide sequence, charge state and amino acid residue position* ** Methionine oxidation*	*Coverage (%)*
3068	210704_data13	**Elongation factor 1-delta, *EF1D_RABIT, P53787***	IASLEVENQNLR, 2+, 84 - 95 LVPVGYGIR, 1+, 232 - 240	8
3141	210704_data15	**Microtubule associated protein, RP/EB family, member 1, *MARE1-HUMAN, Q15691***	LEHEYIQNFK, 1+, 66 - 75 ILQAGFK, 1+, 76 - 82 IIPVDK, 1+, 89 - 94 LTVEDLEKER, 1+, 204 - 213 DFYFGK, 1+, 214 - 219	15
2307	220704_data10	**Stress-70 protein, *GRP75_CRIGR, O35501***	TTPSVVAFTADGER, 1+, 86 - 99 VINEPTAAALAYGLDK, 2+, 219 - 234 AQFEGIVTDLIK, 1+, 349 - 360 VQQTVQDLFGR, 1+, 395 - 405 SQVFSTAADGQTQVEIK, 2+, 469 - 485 QAASSLQQASLK, 1+, 635 - 646	12
2308	220704_data11	**Heat shock cognate 71 kDa protein, *HSP7C_CRIGR, P19378***	MKEIAEAYLGK, 1+, 127 - 137 DAGTIAGLNVLR, 1+, 160 - 171 GTLDPVEK, 1+, 312 - 319 LLQDFFNGK, 1+, 349 - 357 DNNLLGK, 1+, 452 - 458 LSKEDIER, 1+, 510 - 517 NSLESYAFNMK, 1+, 540 – 550	10

Proteins identified in CHO-K1 2-D gels cont.

Spot ID number	*File name*	*Protein ID*	*peptide sequence, charge state and amino acid residue position* ** Methionine oxidation*	*Coverage (%)*
3862	220704_data12	**Heat shock cognate 71 kDa protein, *HSP7C_CRIGR, P19378***	TTPSYVAFTDTER, 2+, 37 - 49 NQVAM*NPTNTVFDAK, 2+, 57 - 71 NQVAMNPTNTVFDAKR, 2+, 57 - 72 VQVEYKGEAK, 1+, 103 - 112 SFYPEEVSSMVLTK, 1+, 113 - 126 MKEIAEAYLGK, 1+, 127 - 137 TVTNAVVTVPAYFNDSQR, 2+, 138 - 155 DAGTIAGLNVLR, 1+, 160 - 171 IINEPTAAAIAYGLDKK, 2+, 172 - 188 STAGDTHLGGEDFDNR, 1+ & 2+, 221 - 236 M*VNHFIAEFK, 1+, 237 - 246 ARFEELNADLFR, 1+, 300 - 311 FEELNADLFR, 1+, 302 - 311 GTLDPVEK, 1+, 312 - 319 DAKLDK, 1+, 323 - 328 LDKSQIHDIVLVGGSTR, 2+, 326 - 342 SQIHDIVLVGGSTR, 1+, 329 - 342 LLQDFFNGK, 1+, 349 - 357 SINPDEAVAYGAAVQAAILSGDK, 2+, 362 - 384 QTQTFTTYSDNQPGVLIQVYEGER, 2+, 424 - 447 AM*TKDNNLLGK, 2+, 448 - 458 DNNLLGK, 1+, 452 - 458 ITITNDK, 1+, 501 - 507 ITITNDKGR, 1+ & 2+, 501 - 509 LSKEDIER, 1+, 510 - 517 NSLESYAFNMK, 1+, 540 - 550 NSLESYAFNM*K, 1+, 540 - 550 LYQSAGGM*PGGMPGGFPGGGAPPSGGASSGPTIEEVD , 2+, 610 - 646	48

Proteins identified in CHO-K1 2-D gels cont.

Spot ID number	*File name*	*Protein ID*	*peptide sequence, charge state and amino acid residue position* ** Methionine oxidation*	*Coverage (%)*
2316	220704_data13	**Heat shock cognate 71 kDa protein, *HSP7C_CRIGR, P19378***	IINEPTAAAIAYGLDKK, 2+, 172 - 188 STAGDTHLGGEDFDNR, 2+, 221 - 236 LLQDFFNGK, 1+, 349 - 357 SINPDEAVAYGAAVQAAILSGDK, 2+, 362 - 384 DNNLLGK, 1+, 452 - 458 ITITNDK, 1+, 501 - 507 NSLESYAFNMK, 1+, 540 - 550	14
2157	220704_data15	**78 kDa glucose regulated protein, *GRP78_HUMAN, P11021***	VEIIANDQGNR, 1+, 50 - 60 NQLTSNPENTVFDAK, 1+ & 2+, 82 - 96 MKETAEAYLGK, 1+, 153 - 163 IINEPTAAAIAYGLDKR, 2+, 198 - 214 ALSSQHQAR, 1+, 298 - 306 STMKPVQK, 1+, 337 - 344 SDIDEIVLVGGSTR, 1+, 354 - 367 IQQLVK, 1+, 371 - 376 EFFNGKEPSR, 1+, 377 - 386 NTVVPTKK, 1+, 440 - 447 SQIFSTASDNQPTVTIK, 2+, 448 - 464 VYEGERPLTK, 1+, 465 - 474 LTPEEIER, 1+, 533 - 540 NELESYAYSLK, 1+, 563 - 573 NQIGDKEK, 1+, 574 - 581 ELEEIVQPIISK, 1+, 622 - 633	27
2179	220704_data16	**78 kDa glucose regulated protein, *GRP78_HUMAN, P11021***	VEIIANDQGNR, 1+, 50 - 60 NQLTSNPENTVFDAK, 2+, 82 - 96 MKETAEAYLGK, 1+, 153 - 163 ALSSQHQAR, 1+, 298 - 306 VLEDSDLK, 1+, 345 - 352 IQQLVK, 1+, 371 - 376 NELESYAYSLK, 1+, 563 – 573	11

Proteins identified in CHO-K1 2-D gels cont.

Spot ID number	*File name*	*Protein ID*	*peptide sequence, charge state and amino acid residue position* ** Methionine oxidation*	*Coverage (%)*
2114	230704_data0 6	**Calreticulin, , *CRTC_CRIGR, Q8K3H7***	HKSDFGK, 1+, 42 - 48 FVLSSGK, 1+, 49 - 55 FYGDQEKDK, 1+, 56 - 64 GLQTSQDAR, 1+, 65 - 73 VHVIFNYK, 1+, 144 - 151 NVLINK, 1+, 154 - 159 QIDNPDYK, 1+, 279 - 286	13
3202	230704_data0 7	**Proteasome subunit alpha type 1, *PSA1_HUMAN, P25786***	THAVLVALK, 1+, 42 - 50 AQSELAAHQK, 1+, 52 - 61 LVSLIGSK, 1+, 108 - 115 ETLPAEQDLTTK, 1+, 197 - 208 NVSIGIVGK, 1+, 209 - 217	18
3337	230704_data0 8	**Peroxiredoxin 4, *PRDX4_HUMAN, Q13162***	SVDETLR, 1+, 224 - 230 LVQAFQYTDK, 1+, 231 - 240	6
2787	260704_data0 4	**Nuclear migration protein nudC (nuclear distribution protein C homolog), *NUDC_RAT, Q63525***	LITQTFNHHNQLAQK, 2+, 54 - 68 AETPGPQIK, 2+, 97 - 105 LKPNLGNGADLPNYR, 2+, 160 - 174 VEESSWLIEDGK, 2+, 229 - 240 LVTSDPEINTK, 2+, 258 - 268 LSDLDSETR, 2+, 277 - 285	21
2775	260704_data0 5	**Actin, aortic smooth muscle, *ACTA_HUMAN, P62736***	GYSFVTTAER, 2+, 199 - 208 SYELPDGQVITIGNER, 2+, 241 - 256 IIAPPER, 1+, 331 - 337	9
2763	260704_data0 7	**Actin, cytoplasmic 1, *ACTB_CRIGR, P48975***	HQGVM*VGMGQK, 2+, 40 - 50 HQGVMVGM*GQK, 2+, 40 - 50 HQGVM*VGM*GQK, 2+, 40 - 50 SYELPDGQVITIGNER, 2+, 239 - 254 DLYANTVLSGGTTM*YPGIADR, 2+, 292 - 312 EITALAPSTMK, 2+, 316 - 326 EITALAPSTM*K, 2+, 316 - 326 QEYDESGPSIVHR, 2+, 360 – 372	19

Proteins identified in CHO-K1 2-D gels cont.

Spot ID number	*File name*	*Protein ID*	*peptide sequence, charge state and amino acid residue position* ** Methionine oxidation*	*Coverage (%)*
2768	260704_data0 8	**Actin, cytoplasmic 1,** ***ACTB_CRIGR, P48975***	HQGVMVGMGQK, 2+, 40 - 50 HQGVM*VGMGQK, 2+, 40 - 50 HQGVMVGM*GQK, 2+, 40 - 50 HQGVM*VGM*GQK, 2+, 40 - 50 TTGIVMDSGDGVTHTVPIYEGYALPHAILR, 3+, 148 - 177 SYELPDGQVITIGNER, 2+, 239 - 254 EITALAPSTMKF, 2+, 316 - 326 EITALAPSTM*KF, 2+, 316 - 326 QEYDESGPSIVHRF360 - 372	22
2776	260704_data0 9	**Actin, cytoplasmic 1,** ***ACTB_CRIGR, P48975***	SYELPDGQVITIGNER, 2+, 239 - 254 QEYDESGPSIVHR, 2+, 360 - 372	8
2769	260704_data1 0	**Actin, cytoplasmic 1,** ***ACTB_CRIGR, P48975***	SYELPDGQVITIGNER, 2+, 239 - 254 EITALAPSTMK, 2+, 316 - 326 QEYDESGPSIVHR, 2+, 360 - 372	11
3026	270704_data0 4	**Elongation factor 1-delta,** ***EF1D_MOUSE, P57776***	QENGASVILR, 2+, 38 - 47 SLAGSSGPGASSGPGGDHSELIVR, 2+, 59 - 82 LVPVGYGIR, 2+, 232 - 240	15
2459	270704_data1 3	**60 kDa heat shock protein,** ***CH60_HUMAN, P10809***	KGVITVK, 1+, 196 - 202 GYISPYFINTSK, 2+, 222 - 233 VGLQVVAVK, 1+, 293 - 301 GIIDPTK, 1+, 517 - 523	6
2674	290704_data0 8	**Protein disulfide isomerase A6,** ***PDIA6_RAT, Q63081***	VGAVNADKHQSLGGQYGVQGFPTIK, 2+, 78 - 102 GFPTIK, 1+, 97 - 102 GFPTIK, 1+, 236 - 241 GSFSEQGINEFLR, 2+, 374 - 386	10
2385	300704_data3 1	**T-complex protein 1 subunit beta,** ***TCPB_HUMAN, P78371***	NIGVDNPAAK, 1+, 72 - 81 GATQQILDEAER, 1+, 376 - 387 VDNIIK, 1+, 516 - 521	5

Appendix F: Publications and acknowledgement letter from Home Office for research conducted

The A P C
Animal Procedures Committee

5th Floor, Hannibal House, Elephant & Castle, London, SW1P 2AW
Direct Line 020 7035 4788 / 4778 / 4777 Fax 020 7035 4776
email: apc.secretariat@homeoffice.gsi.gov.uk web-site: www.apc.gov.uk

Our Ref
Your Ref

03 April 2006

Dr D L Xing
Division of Bacteriology
NIBSC
Blanche Lane
South Mimms
Potters Bar
Hertfordshire EN6 3QG

Dear Dr Xing,

Development of an alternative test to the histamine challenge test based on in vitro enzymatic-HLPC coupled assay for pertussis vaccines

Thank you forwarding the final report on the above project earlier this year. This was discussed at the last meeting of the Research and Alternatives sub-committee and members were very pleased with the successful results of the project highlighted by the posters and literature produced.

Members would be interested if you could keep them informed of any further developments of alternative methods arising from this project.

Yours sincerely

Philip Brenner
APC Secretariat

Ad Hoc group. (1988) Placebo controlled trial of two acellular pertussis vaccines in Sweden. *Lancet.* **I**: 955-966.

Ahmad, S., Ahuja, R., Venner, T. J., & Gupta, R. S. (1990). Identification of a protein altered in mutants resistant to microtubule inhibitors as a member of the major heat shock protein (hsp70) family. *Molecular and Celullar Biology.* **10**: 5160-5165.

Akama, K., Kameyama, S., Otani, S., Sadahiro, S., & Murata, R. (1971) Reversion of toxicity of diphtheria toxoid. *Japanese Journal of Medicine. Science and Biology.* **24**: 183-187.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). Chapter 18, The Mechanics of Cell Division. In: Molecular Biology of the Cell 4th edition, Garland Science, New York.

Alexandrova, A. Y., Kopnin, P. B., Vasiliev, J. M., & Kopnin, B. P. (2006) ROS up-regulation mediates Ras-induced changes of cell morphology and motility. *Experimental Cell Research.* **312**: 2066-2073.

Alfano, M., Pushkarsky, T., Poli, G., & Bukrinsky, M. (2000) The B-oligomer of pertussis toxin inhibits human immunodeficiency virus type 1 replication at multiple stages. *Journal of Virology.* **74**: 8767-8770.

Alfano, M., Vallanti, G., Biswas, P., Bovolenta, C., Vicenzi, E., Mantelli, B., Pushkarsky, T., Rappuoli, R., Lazzarin, A., Bukrinsky, M., & Poli, G. (2001) The binding subunit of pertussis toxin inhibits HIV replication in human macrophages and virus expression in chronically infected promonocytic U1 cells. *Journal of Immunology.* **166**: 1863-1870.

Allen, A. & Maskell, D. (1996) The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in *Bordetella pertussis*. *Molecular Microbiology.* **19**: 37-52.

Allen, A. G., Thomas, R. M., Cadisch, J. T., & Maskell, D. J. (1998) Molecular and functional analysis of the lipopolysaccharide biosynthesis locus wlb from *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. *Molecular Microbiology.* **29**: 27-38.

Anderson, L. & Seilhamer, J. (1997) A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis.* **18**: 533-537.

Andoh, T., Chock, P. B., & Chiueh, C. C. (2002) The roles of thioredoxin in protection against oxidative stress-induced apoptosis in SH-SY5Y cells. *Journal of Biological Chemistry.* **277**: 9655-9660.

Anonymous. (2004) Hexavalent vaccination and sudden death. *Prescrire international.* **13**: 59.

Apweiler, R., Bairoch, A., & Wu C.H. (2004) Protein sequence databases. *Current Opinion in Chemical Biology.* **8**: 76-80

Armstrong, G. D., Howard, L. A., & Peppler, M. S. (1988) Use of glycosyltransferases to restore pertussis toxin receptor activity to asialoagalactofetuin. *Journal of Biological Chemistry.* **263**: 8677-8684.

Armstrong, G. D., Clark, C. G., & Heerze, L. D. (1994) The 70-kilodalton pertussis toxin-binding protein in Jurkat cells. *Infection and Immununity.* **62**: 2236-2243.

Arner, E. S. & Holmgren, A. (2000) Physiological functions of thioredoxin and thioredoxin reductase. *European Journal of Biochemistry.* **267**: 6102-6109.

Ausiello, C. M., Lande, R., Stefanelli, P., Fazio, C., Fedele, G., Palazzo, R., Urbani, F., & Mastrantonio, P. (2003) T-cell immune response assessment as a complement to serology and intranasal protection assays in determining the protective immunity induced by acellular pertussis vaccines in mice. *Clinical and Diagnostic Laboratory Immunology.* **10**: 637-642.

Baggerman, G., Vierstraete, Evy., Loof, De Loof, A., & Schoofs, L. (2005) Gel-Based Versus Gel-Free Proteomics: A review. Combinatorial Chemistry & High Thoroughput Screening. **8** :669-677.

Bairoch, A., Apweiler, R., Wu, C.H., Barker, W.C., Boeckmann, B., Ferro, S., Gasteiger, E., Huang, H., Lopez, Rodrigo, L., Magrane, M., Martin, M.J., Natale, D.A., O'Donovan, C., Redaschi, N., & Yeh, L-S. (2005) The universal protein resource (UniProt). *Nucleic Acids Research.* **33**: D154-D159.

Ben Nun, A., Mendel, I., & Kerlero, d. R. (1997) Immunomodulation of murine experimental autoimmune encephalomyelitis by pertussis toxin: the protective activity, but not the disease-enhancing activity, can be attributed to the nontoxic B-oligomer. *Proceedings of the Association of American Physicians.* **109**: 120-125.

Bentsi-Enchill, A. D., Halperin, S. A., Scott, J., MacIsaac, K., & Duclos, P. (1997) Estimates of the effectiveness of a whole-cell pertussis vaccine from an outbreak in an immunized population. *Vaccine.* **15**: 301-306.

Bergman, R. K. & Munoz, J. (1966) Protection against histamine shock by catecholamines in *Bordetella pertussis*-treated, adrenalectomized, or adrenergic blocked mice. *Proceedings of the Society for Experimental Biology and Medicine.* **122**: 428-433.

Bergman, R. K. & Munoz, J. (1968) Efficacy of beta-adrenergic blocking agents in inducing histamine sensitivity in mice. *Nature.* **217**: 1173-1174.

Bhat, V. B., Choi, M. H., Wishnok, J. S., & Tannenbaum, S. R. (2005) Comparative plasma proteome analysis of lymphoma-bearing SJL mice. *Journal of Proteome Research.* **4**: 1814-1825.

Bierfreund, U., Lemm, T., Hoffmann, A., Uhlhorn-Dierks, G., Childs, R. A., Yuen, C. T., Feizi, T., & Sandhoff, K. (1999) Recombinant GM2-activator protein stimulates in vivo degradation of GA2 in GM2 gangliosidosis AB variant fibroblasts but exhibits no detectable binding of GA2 in an in vitro assay. *Neurochemical Research.* **24**: 295-300.

Bischoff, F. R., Krebber, H., Smirnova, E., Dong, W., & Ponstingl, H. (1995) Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. *EMBO Journal.* **14**: 705-715.

Black, S. & Greenberg, D. P. (2005) A combined diphtheria, tetanus, five-component acellular pertussis, poliovirus and *Haemophilus influenzae* type b vaccine. *Expert Review of Vaccines.* **4**: 793-805.

Blanke, S.R. (2006) Portals and pathways: principals of bacterial toxin entry into host Cells. Microbe. **1**: 26-32.

Bordet, J & Gengou, O. (1906) Le microbe de la coqueluche. *Annales Institute Pasteur (Paris).* **20**: 731-741.

Braun, M. M., Terracciano, G., Salive, M. E., Blumberg, D. A., Vermeer-de Bondt, P. E., Heijbel, H., Evans, G., Patriarca, P. A., & Ellenberg, S. S. (1998) Report of a US public health service workshop on hypotonic-hyporesponsive episode (HHE) after pertussis immunization. *Pediatrics.* **102**: E52.

Brennan, M. J., David, J. L., Kenimer, J. G., & Manclark, C. R. (1988) Lectin-like binding of pertussis toxin to a 165-kilodalton Chinese hamster ovary cell glycoprotein. *Journal of Biological Chemistry.* **263**: 4895-4899.

Brito, G. A., Souza, M. H., Melo-Filho, A. A., Hewlett, E. L., Lima, A. A., Flores, C. A., & Ribeiro, R. A. (1997) Role of pertussis toxin A subunit in neutrophil migration and vascular permeability *Infection and Immunity.* **65**: 1114-1118.

Bruckener, K. E., el Baya, A., Galla, H. J., & Schmidt, M. A. (2003) Permeabilization in a cerebral endothelial barrier model by pertussis toxin involves the PKC effector pathway and is abolished by elevated levels of cAMP. *Journal of Cell Science.* **116**: 1837-1846.

Burns, D. L., Kenimer, J. G., & Manclark, C. R. (1987) Role of the A subunit of pertussis toxin in alteration of Chinese hamster ovary cell morphology. *Infection and Immunity.* **55**: 24-28.

Burns, D. L. & Manclark, C. R. (1989) Role of cysteine 41 of the A subunit of pertussis toxin. *Journal of Biological Chemistry.* **264**: 564-568.

Burns, D. L. (2003) Type IV transporters of pathogenic bacteria. *Current Opinion in Microbiology.* **6**: 29-34.

Canthaboo, C., Xing, D., & Corbel, M. (1999) Development of a nitric oxide induction assay as a potential replacement for the intracerebral mouse protection test for potency assay of pertussis whole cell vaccines. *Developments in Biological Standardization.* **101**: 95-103.

Canthaboo, C., Williams, L., Xing, D. K., & Corbel, M. J. (2000) Investigation of cellular and humoral immune responses to whole-cell and acellular pertussis vaccines *Vaccine*, **19**: 637-643.

Canthaboo, C., Xing, D., Douglas, A., & Corbel, M. (2000) Investigation of an aerosol challenge model as alternative to the intracerebral mouse protection test for potency assay of whole cell pertussis vaccines. *Biologicals*. **28**: 241-246.

Canthaboo, C., Xing, D., Wei, X. Q., & Corbel, M. J. (2002) Investigation of role of nitric oxide in protection from *Bordetella pertussis* respiratory challenge. *Infection and Immununity*. **70**: 679-684.

Capiau, C., Petre, J., Van Damme, J., Puype, M., & Vandekerckhove, J. (1986) Protein-chemical analysis of pertussis toxin reveals homology between the subunits S2 and S3, between S1 and the A chains of enterotoxins of *Vibrio cholerae* and *Escherichia coli* and identifies S2 as the haptoglobin-binding subunit. *FEBS Letters*. **204**: 336-340.

Capiau, C., Poolman, J., Hoet, B., Bogaerts, H., & Andre, F. (2003) Development and clinical testing of multivalent vaccines based on a diphtheria-tetanus-acellular pertussis vaccine: difficulties encountered and lessons learned. *Vaccine*. **21**: 2273-2287.

Carbonetti, N. H., Irish, T. J., Chen, C. H., O'Connell, C. B., Hadley, G. A., McNamara, U., Tuskan, R. G., & Lewis, G. K. (1999) Intracellular delivery of a cytolytic T-lymphocyte epitope peptide by pertussis toxin to major histocompatibility complex class I without involvement of the cytosolic class I antigen processing pathway. *Infection and Immunity*. **67**: 602-607.

Caroff, M., Brisson, J., Martin, A., & Karibian, D. (2000) Structure of the Bordetella pertussis 1414 endotoxin. *FEBS Letters*. **477**: 8-14.

Casey, J. R. & Pichichero, M. E. (2005) Acellular pertussis vaccine safety and efficacy in children, adolescents and adults. *Drugs*. **65**: 1367-1389.

Castro, M. G., McNamara, U., & Carbonetti, N. H. (2001) Expression, activity and cytotoxicity of pertussis toxin S1 subunit in transfected mammalian cells. *Cellular Microbiology*. **3**: 45-54.

Centers for Disease Control and Prevention (CDC) (2005a) Recommended antimicrobial agents for the treatment and postexposure prophylaxis of pertussis. *Morbidity and Mortality Weekly Reports*. **54**: 1-16.

CDC (2005b). Pertussis—United States, 2001–2003. *Morbidity and Mortality Weekly Reports* **54**:1283–1286.

CDC (2006) Pertussis. Available at:
http://www.cdc.gov/niP/publications/pink/pert.pdf#search=%22CDC%202006%20and%20pertussis%20epidemiology%22

Celentano, L. P., Massari, M., Paramatti, D., Salmaso, S., & Tozzi, A. E. (2005) Resurgence of pertussis in Europe. *Pediatrics Infectious Diseases Journal*. **24**: 761-765.

Champion, K. M., Arnott, D., Henzel, W. J., Hermes, S., Weikert, S., Stults, J., Vanderlaan, M., & Krummen, L. (1999) A two-dimensional protein map of Chinese hamster ovary cells. *Electrophoresis* **20**: 994-1000.

Charlwood, J., Clayton, P., Keir, G., Mian, N., & Winchester, B. (1998) Defective galactosylation of serum transferrin in galactosemia. *Glycobiology*. **8** : 351-357.

Chen, F., Wollmer, M. A., Hoerndli, F., Munch, G., Kuhla, B., Rogaev, E. I., Tsolaki, M., Papassotiropoulos, A., & Gotz, J. (2004) Role for glyoxalase I in Alzheimer's disease. *Proceedings of the National Academy of Sciences of the USA*. **101**: 7687-7692.

Cherry, J.D, Brunnel, P.A., Golden, G.S. & Karzon, D.T. (1988) Report on the task force on pertussis and pertussis immunisation. Pediatrics. **81**: 939-984.

Cherry, J. D. (1997) Comparative efficacy of acellular pertussis vaccines: an analysis of recent trials. *Pediatrics Infectious Diseases Journal*. **16**: S90-S96.

Cherry, J. D., Gornbein, J., Heininger, U., & Stehr, K. (1998) A search for serologic correlates of immunity to *Bordetella pertussis* cough illnesses. *Vaccine* **16**: 1901-1906.

Cherry, J. D. (1999) Pertussis in the preantibiotic and prevaccine era, with emphasis on adult pertussis. *Clinical Infectious Diseases*. **28**: S107-S111.

Cherry, J. D., Xing, D. X., Newland, P., Patel, K., Heininger, U., & Corbel, M. J. (2004) Determination of serum antibody to *Bordetella pertussis* adenylate cyclase toxin in vaccinated and unvaccinated children and in children and adults with pertussis. *Clinical Infectious Diseases*. **38**: 502-507.

Chevallet, M., Wagner, E., Luche, S., van Dorsselaer, A., Leize-Wagner, E., & Rabilloud, T. (2003) Regeneration of peroxiredoxins during recovery after oxidative stress: only some overoxidized peroxiredoxins can be reduced during recovery after oxidative stress. *Journal of Biological Chemistry*. **278**: 37146-37153.

Chevallet, M., Diemer, H., Luche, S., van Dorsselaer, A., Rabilloud, T., & Leize-Wagner, E. (2006), Improved mass spectrometry compatibility is afforded by ammoniacal silver staining. *Proteomics*. **6**: 2350-2354.

Chiu, C. P. & Harley, C. B. (1997) Replicative senescence and cell immortality: the role of telomeres and telomerase. *Proceedings of the Society for Experimental Biology and Medicine*. **214**: 99-106.

Chiu, Y. H., Xiang, X., Dawe, A. L., & Morris, N. R. (1997) Deletion of nudC, a nuclear migration gene of *Aspergillus nidulans*, causes morphological and cell wall abnormalities and is lethal. *Molecular Biology of the Cell*. **8**: 1735-1749.

Choi, M. H., Lee, I. K., Kim, G. W., Kim, B. U., Han, Y. H., Yu, D. Y., Park, H. S., Kim, K. Y., Lee, J. S., Choi, C., Bae, Y. S., Lee, B. I., Rhee, S. G., & Kang, S. W. (2005) Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II. *Nature*. **435**: 347-353.

Ciechanover, A. (1998) The ubiquitin-proteasome pathway: on protein death and cell life. *EMBO Journal.* **17**: 7151-7160.

Clark, C. G. & Armstrong, G. D. (1990) Lymphocyte receptors for pertussis toxin. *Infection and Immunity.* **58**: 3840-3846.

Coates, P. J., Nenutil, R., McGregor, A., Picksley, S. M., Crouch, D. H., Hall, P. A., & Wright, E. G. (2001) Mammalian prohibitin proteins respond to mitochondrial stress and decrease during cellular senescence. *Experimental Cell Research.* **265**: 262-273.

Cody, C. L., Baraff, L. J., Cherry, J. D., Marcy, S. M., & Manclark, C. R. (1981) Nature and rates of adverse reactions associated with DTP and DT immunizations in infants and children. *Pediatrics.* **68**: 650-660.

Confer, D. L. & Eaton, J. W. (1982) Phagocyte impotence caused by an invasive bacterial adenylate cyclase. *Science.* **217**: 948-950.

Cookson, B. T., Tyler, A. N., & Goldman, W. E. (1989) Primary structure of the peptidoglycan-derived tracheal cytotoxin of *Bordetella pertussis*. *Biochemistry.* **28**: 1744-1749.

Coote, J. G. (1996) The RTX toxins of Gram-negative bacterial pathogens: modulators of the host immune system. *Reviews in Medical Microbiology.* **7**: 53-62.

Corbel, M. J. & Xing, D. K. (2004). Toxicity and potency evaluation of pertussis vaccines. *Expert Review of Vaccines.* **3**: 89-101.

Corbel, M. J., Kreeftenberg, J. G., & Knezevic, I. (2004) WHO Working Group on the standardisation and control of pertussis vaccines-report of a meeting held on 6-7 May 2003, Ferney Voltaire, France. *Vaccine.* **22**: 293-300.

Coutte, L., Antoine, R., Drobecq, H., Locht, C., & Jacob-Dubuisson, F. (2001) Subtilisin-like autotransporter serves as maturation protease in a bacterial secretion pathway. *EMBO Journal.* **20**: 5040-5048.

Craig-Mylius, K. A., Stenson, T. H., & Weiss, A. A. (2000) Mutations in the S1 subunit of pertussis toxin that affect secretion. *Infection and Immunity.* **68**: 1276-1281.

Cryz, S. J., Jr., Friedman, R. L., Pavlovskis, O. R., & Iglewski, B. H. (1981) Effect of formalin toxoiding on Pseudomonas aeruginosa toxin A: biological, chemical, and immunochemical studies. *Infection and Immunity.* **32**: 759-768.

Cyr, T., Menzies, A. J., Calver, J., & Whitehouse, L. W. (2001) A quantitative analysis for the ADP-ribosylation activity of pertussis toxin: an enzymatic-HPLC coupled assay applicable to formulated whole cell and acellular pertussis vaccine products. *Biologicals.* **29**: 81-95.

Day, P. J., Owens, S. R., Wesche, J., Olsnes, S., Roberts, L. M., & Lord, J. M. (2001) An interaction between ricin and calreticulin that may have implications for toxin trafficking. *Journal of Biological Chemistry.* **276**: 7202-7208.

de Melker, H. E., Schellekens, J. F., Neppelenbroek, S. E., Mooi, F. R., Rumke, H. C., & Conyn-van Spaendonck, M. A. (2000) Reemergence of pertussis in the highly vaccinated population of the Netherlands: observations on surveillance data. *Emerging Infectious Diseases.* **6**: 348-357.

de Wildt, D. J., Kreeftenberg, H. G., & Nijkamp, F. P. (1983) Impaired autonomic responsiveness of the cardiovascular system of the rat induced by a heat-labile component of *Bordetella pertussis* vaccine. *Infection and Immunity.* **41**: 476-481.

Decker, M. D., Edwards, K. M., Steinhoff, M. C., Rennels, M. B., Pichichero, M. E., Englund, J. A., Anderson, E. L., Deloria, M. A., & Reed, G. F. (1995) Comparison of 13 acellular pertussis vaccines: adverse reactions. *Pediatrics.* **96**: 557-566.

Denoel, P., Poolman, J., Carletti, G., & Veitch, K. (2002) Effects of adsorption of acellular pertussis antigens onto different aluminium salts on the protective activity in an intranasal murine model of *Bordetella pertussis* infection. *Vaccine.* **20**: 2551-2555.

Di Fiore, B., Ciciarello, M., Mangiacasale, R., Palena, A., Tassin, A. M., Cundari, E., & Lavia, P. (2003) Mammalian RanBP1 regulates centrosome cohesion during mitosis *Journal of Cell Science.* **116**: 3399-3411.

Donnelly, S., Loscher, C. E., Lynch, M. A., & Mills, K. H. (2001) Whole-cell but not acellular pertussis vaccines induce convulsive activity in mice: evidence of a role for toxin-induced interleukin-1beta in a new murine model for analysis of neuronal side effects of vaccination. *Infection and Immunity.* **69**: 4217-4223.

Dubois, M., Hamilton, J.K., Rebers, P.A., & Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry.* 28: 350-356.

Edwards, K. M., Meade, B. D., Decker, M. D., Reed, G. F., Rennels, M. B., Steinhoff, M. C., Anderson, E. L., Englund, J. A., Pichichero, M. E., & Deloria, M. A. (1995) Comparison of 13 acellular pertussis vaccines: overview and serologic response. *Pediatrics.* **96**: 548-557.

Egeo, A., Mazzocco, M., Arrigo, P., Vidal-Taboada, J. M., Oliva, R., Pirola, B., Giglio, S., Rasore-Quartino, A., & Scartezzini, P. (1998) Identification and characterization of a new human gene encoding a small protein with high homology to the proline-rich region of the SH3BGR gene. *Biochemical and Biophysical Research Communication.* **247**: 302-306.

el Baya, A., Linnemann, R., Olleschik-Elbheim, L., Robenek, H., & Schmidt, M. A. (1997a) Endocytosis and retrograde transport of pertussis toxin to the Golgi complex as a prerequisite for cellular intoxication. *European Journal of Cell Biology.* **73**: 40-48.

el Baya, A., Linnermann, R., Olleschik-Elbheim, L., & Schmidt, M. A. (1997b) Pertussis toxin. Entry into cells and enzymatic activity. *Advances in Experimental Medicine and Biology.* **419**: 83-86.

el Baya, A., Bruckener, K., & Schmidt, M. A. (1999) Nonrestricted differential intoxication of cells by pertussis toxin. *Infection and Immunity.* **67**: 433-435.

Emsley, P., McDermott, G., Charles, I. G., Fairweather, N. F., & Isaacs, N. W. (1994) Crystallographic characterization of pertactin, a membrane-associated protein from *Bordetella pertussis*. *Journal of Molecular Biology*. **235**: 772-773.

European Pharmacopoeia (2005) Diptheria, Tetanus and Pertussis Vaccine (Adsorbed). In: European Pharmacopoeia, 5.0. Strasbourg: Council of Europe. pg. 643-644.

European Pharmacopoeia (2006) Diptheria, Tetanus and Pertussis (Acellular, component) Vaccine (Adsorbed). In: European Pharmacopoeia 5.4 Strasbourg: Council of Europe. pg 3851-3852.

Everse, J., Lappi, D. A., Beglau, J. M., Lee, C. L., & Kaplan, N. O. (1977) Investigations into the relationship between structure and function of diphtheria toxin. *Proceedings of the National Academy of the Sciences of the USA*. **74**: 472-476.

Farizo, K. M., Cafarella, T. G., & Burns, D. L. (1996) Evidence for a ninth gene, ptlI, in the locus encoding the pertussis toxin secretion system of *Bordetella pertussis* and formation of a PtlI-PtlF complex. *Journal of Biological Chemistry*. **271**: 31643-31649.

Farizo, K. M., Huang, T., & Burns, D. L. (2000) Importance of holotoxin assembly in Ptl-mediated secretion of pertussis toxin from *Bordetella pertussis*. *Infection and Immunity*. **68**: 4049-4054.

Farizo, K. M., Fiddner, S., Cheung, A. M., & Burns, D. L. (2002) Membrane localization of the S1 subunit of pertussis toxin in *Bordetella pertussis* and implications for pertussis toxin secretion. *Infection and Immunity*. **70**: 1193-1201.

Feizi, T., Stoll, M. S., Yuen, C. T., Chai, W., & Lawson, A. M. (1994) Neoglycolipids: probes of oligosaccharide structure, antigenicity, and function. *Methods in Enzymology*. **230**: 484-519.

Ferens-Sieczkowska, M. & Olczak, M. (2001) Carbohydrate structures of haptoglobin in sera of healthy people and a patient with congenital disorder of glycosylation. *Zeitschrift für Naturforschung. C, Journal of biosciences*. **56**: 122-131.

Fernandez, R. C. & Weiss, A. A. (1994) Cloning and sequencing of a *Bordetella pertussis* serum resistance locus. *Infection and Immunity*. vol. **62**: 4727-4738.

Fernandez, R. C. & Weiss, A. A. (1996) Susceptibilities of *Bordetella pertussis* strains to antimicrobial peptides. *Antimicrobial agents and Chemotherapy*. **40**: 1041-1043.

FDA (2006) Code of Federal Regulations. Food and Drugs, Part 610, General Biological Products Standards. Washington, DC: US Government Printing Office **7**: 65–90. Also available at http://frwebgate2.access.gpo.gov/cgibin/waisgate.cgi?WAISdocID=745700108144+1+0+0&WAISaction=retrieve

Fiaschi, T., Cozzi, G., Raugei, G., Formigli, L., Ramponi, G., & Chiarugi, P. (2006) Redox regulation of beta-actin during integrin-mediated cell adhesion. *Journal of Biological Chemistry*. **281**: 22983-22991.

Finck-Barbancon, V. & Barbieri, J. T. (1995) ADP-ribosylation of alpha i3C20 by the S1 subunit and deletion peptides of S1 of pertussis toxin. *Biochemistry.* **34**: 1070-1075.

Finn, T. M. & Stevens, L. A. (1995) Tracheal colonization factor: a *Bordetella pertussis* secreted virulence determinant. *Molecular Microbiology.* **16**: 625-634.

Finney, D.J. (1978) Statistical Method in Biological Assay, Charles Griffin, London.

Fishel, C. W., Szentivanyi, A., & Talmage, D.W. (1962) Sensitization and desensitization of mice to histamine and serotonin by neurohumors. *Journal of Immunology.* **89**: 8-18.

Flak, T. A. & Goldman, W. E. (1999) Signalling and cellular specificity of airway nitric oxide production in pertussis. *Cellular Microbiology.* **1**: 51-60.

Flak, T. A., Heiss, L. N., Engle, J. T., & Goldman, W. E. (2000) Synergistic epithelial responses to endotoxin and a naturally occurring muramyl peptide. *Infection and Immunity.* **68**: 1235-1242.

Forsyth, K., Tan, T., von Konig, C. H., Caro, J. J., & Plotkin, S. (2005) Potential strategies to reduce the burden of pertussis. *Pediatrics Infectious Diseases Journal.* **24**: S69-S74.

Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vliegenthart, F. G., Binette, J. P., & Schmid, K. (1978) Determination of the primary structures of 16 asialo-carbohydrate units derived from human plasma alpha 1-acid glycoprotein by 360-MHZ 1H NMR spectroscopy and permethylation analysis. *Biochemistry.* **17**: 5206-5214.

Fowler, S., Byron, O., Jumel, K., Xing, D., Corbel, M. J., & Bolgiano, B. (2003) Novel configurations of high molecular weight species of the pertussis toxin vaccine component. *Vaccine.* **21**: 2678-2688.

Fowler, S., Xing, D. K., Bolgiano, B., Yuen, C. T., & Corbel, M. J. (2003) Modifications of the catalytic and binding subunits of pertussis toxin by formaldehyde: effects on toxicity and immunogenicity. *Vaccine.* **21**: 2329-2337.

Fraenkel-Conrat, H., Olcott, H.S. (1948) The reaction of Formaldehyde with proteins. V. Cross-linking between Amino and Primary Amide or Guanidyl Groups. *Journal of American Chemical Society.* **70**: 2673-2684.

Frickel, E. M., Riek, R., Jelesarov, I., Helenius, A., Wuthrich, K., & Ellgaard, L. (2002) TROSY-NMR reveals interaction between ERp57 and the tip of the calreticulin P-domain. *Proceedings of the National Academy of the Sciences of the USA.* **99**: 1954-1959.

Frickel, E. M., Frei, P., Bouvier, M., Stafford, W. F., Helenius, A., Glockshuber, R., & Ellgaard, L. (2004) ERp57 is a multifunctional thiol-disulfide oxidoreductase *Journal of Biological Chemistry.* **279**: 18277-18287.

Friedman, R. L., Fiederlein, R. L., Glasser, L., & Galgiani, J. N. (1987) *Bordetella pertussis* adenylate cyclase: effects of affinity-purified adenylate cyclase on human polymorphonuclear leukocyte functions. *Infection and Immunity.* **55**: 135-140.

Friedman, R. L., Nordensson, K., Wilson, L., Akporiaye, E. T., & Yocum, D. E. (1992) Uptake and intracellular survival of *Bordetella pertussis* in human macrophages. *Infection and Immunity.* **60**: 4578-4585.

Fukui, S., Feizi, T., Galustian, C., Lawson, A. M., & Chai, W. (2002) Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions. *Nature Biotechnology.* **20**: 1011-1017.

Fusaro, G., Dasgupta, P., Rastogi, S., Joshi, B., & Chellappan, S. (2003) Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. *Journal of Biological Chemistry.* **278**: 47853-47861.

Garcia, J. G., Wang, P., Schaphorst, K. L., Becker, P. M., Borbiev, T., Liu, F., Birukova, A., Jacobs, K., Bogatcheva, N., & Verin, A. D. (2002) Critical involvement of p38 MAP kinase in pertussis toxin-induced cytoskeletal reorganization and lung permeability. *The FASEB Journal.* **16**: 1064-1076.

Geuijen, C. A., Willems, R. J., & Mooi, F. R. (1996) The major fimbrial subunit of *Bordetella pertussis* binds to sulfated sugars. *Infection and Immunity.* **64**: 2657-2665.

Geuijen, C. A., Willems, R. J., Bongaerts, M., Top, J., Gielen, H., & Mooi, F. R. (1997) Role of the *Bordetella pertussis* minor fimbrial subunit, FimD, in colonization of the mouse respiratory tract. *Infection and Immunity.* **65**: 4222-4228.

Gillenius, P., Jaatmaa, E., Askelof, P., Granstrom, M., & Tiru, M. (1985) The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese hamster ovary cells. *Journal of Biological Standardization.* **13**: 61-66.

Glaser, P., Danchin, A., Ladant, D., Barzu, O., & Ullmann, A. (1988) *Bordetella pertussis* adenylate cyclase: the gene and the protein. *The Tokai Journal of Experimental and Clinical Medicine.* **13**: 239-252.

Godfroid, F., Denoel, P., & Poolman, J. (2005) Are vaccination programs and isolate polymorphism linked to pertussis re-emergence? *Expert Review of Vaccines.* **4**: 757-778.

Gold, M. S., Noonan, S., Osbourn, M., Precepa, S., & Kempe, A. E. (2003) Local reactions after the fourth dose of acellular pertussis vaccine in South Australia. *The Medical Journal of Australia.* **179**: 191-194.

Goldman, W. E. & Herwaldt, L. A. (1985) *Bordetella pertussis* tracheal cytotoxin. *Developments in Biological Standardization.* **61**: 103-111.

Gorg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., & Weiss, W. (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis.* **21**: 1037-1053.

Gray, L. S., Huber, K. S., Gray, M. C., Hewlett, E. L., & Engelhard, V. H. (1989) Pertussis toxin effects on T lymphocytes are mediated through CD3 and not by pertussis toxin catalyzed modification of a G protein. *Journal of Immunology.* **142**: 1631-1638.

Greco, D., Salmaso, S., Mastrantonio, P., Giuliano, M., Tozzi, A. E., Anemona, A., Ciofi degli Atti, M. L., Giammanco, A., Panei, P., Blackwelder, W. C., Klein, D. L., & Wassilak, S. G. (1996) A controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. Progetto Pertosse Working Group. *New England Journal of Medicine.* **334**: 341-348.

Green, P. J., Tamatani, T., Watanabe, T., Miyasaka, M., Hasegawa, A., Kiso, M., Yuen, C. T., Stoll, M. S., & Feizi, T. (1992) High affinity binding of the leucocyte adhesion molecule L-selectin to 3'-sulphated-Le(a) and -Le(x) oligosaccharides and the predominance of sulphate in this interaction demonstrated by binding studies with a series of lipid-linked oligosaccharides. *Biochemical and Biophysical Research Communication.* **188**: 244-251.

Green, R. A., Wollman, R., & Kaplan, K. B. (2005) APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. *Molecular Biology of the Cell.* **16**: 4609-4622.

Greenberg, D. P. (2005) Pertussis in adolescents: increasing incidence brings attention to the need for booster immunization of adolescents. *Pediatrics Infectious Diseases Journal,* **24**: 721-728.

Grenier-Brossette, N., Bourget, I., Breittmayer, J. P., Ferrua, B., Fehlmann, M., & Cousin, J. L. (1991) Pertussis toxin-induced mitogenesis in human T lymphocytes *Immunopharmacology.* **21**: 109-119.

Griffiths, E. (1988) Efficacy of Whole-Cell Pertussis Vaccine, pg 353-374, In: Wardlaw, A.C. & Parton, P. (Eds.), Pathogenesis and Immunity in Pertussis, John Wiley and Sons Ltd, Chichester, UK.

Guarguaglini, G., Renzi, L., D'Ottavio, F., Di Fiore, B., Casenghi, M., Cundari, E., & Lavia, P. (2000) Regulated Ran-binding protein 1 activity is required for organization and function of the mitotic spindle in mammalian cells in vivo *Cell Growth and Differentiation.* **11**: 455-465.

Guedin, S., Willery, E., Tommassen, J., Fort, E., Drobecq, H., Locht, C., & Jacob-Dubuisson, F. (2000) Novel topological features of FhaC, the outer membrane transporter involved in the secretion of the *Bordetella pertussis* filamentous hemagglutinin. *Journal of Biological Chemistry.* **275**: 30202-30210.

Guermonprez, P., Khelef, N., Blouin, E., Rieu, P., Ricciardi-Castagnoli, P., Guiso, N., Ladant, D., & Leclerc, C. (2001) The adenylate cyclase toxin of *Bordetella pertussis* binds to target cells via the alpha(M)beta(2) integrin (CD11b/CD18). *Journal of Experimental Medicine.* **193**: 1035-1044.

Guiso, N., Szatanik, M., & Rocancourt, M. (1991) Protective activity of *Bordetella* adenylate cyclase-hemolysin against bacterial colonization. *Microbial Pathogenesis.* vol. **11**: 423-431.

Guiso, N., Capiau, C., Carletti, G., Poolman, J., & Hauser, P. (1999) Intranasal murine model of *Bordetella pertussis* infection. I. Prediction of protection in human infants by acellular vaccines. *Vaccine.* **17**: 2366-2376.

Gupta, R. K., Sharma, S. B., Ahuja, S., & Saxena, S. N. (1987) Glutaraldehyde inactivated pertussis vaccine: a less histamine sensitizing vaccine. *Journal of Biological Standardization.* **15**: 159-164.

Gupta, R. K., Sharma, S. B., Ahuja, S., & Saxena, S. N. (1987) The effects of different inactivating agents on the potency, toxicity and stability of pertussis vaccine. *Journal of Biological Standardization.* **15**: 87-98.

Gupta, R. K., Saxena, S. N., Sharma, S. B., & Ahuja, S. (1990) Protection of mice inoculated with purified pertussis toxin and filamentous haemagglutinin against intracerebral challenge with live Bordetella pertussis. *Vaccine.* **8**: 289.

Gupta, R. K., Saxena, S. N., Sharma, S. B., & Ahuja, S. (1991) Production of a safe, potent and immunogenic partially purified acellular pertussis vaccine using simple indigenous techniques. *Developments in Biological Standardization.* **73**: 205-222.

Gustafsson, L., Hallander, H. O., Olin, P., Reizenstein, E., & Storsaeter, J. (1996) A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. *New England Journal of Medicine.* **334**: 349-355.

Gygi, S. P., Rochon, Y., Franza, B. R., & Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast. *Molecular and Cellular Biology.* **19**: 1720-1730.

Gzyl, A., Augustynowicz, E., Rabczenko, D., Gniadek, G., & Slusarczyk, J. (2004) Potency of pertussis component in the DTP vaccine--an overview of three decade study in Poland. *Biologicals.* **32**: 129-137.

Habeeb, A. J. & Hiramoto, R. (1968) Reaction of proteins with glutaraldehyde. *Archives of Biochemistry and Biophysics.* **126**: 16-26.

Halsey, N. A. (2001) Combination vaccines: defining and addressing current safety concerns. *Clinical Infectious Diseases.* **33**: S312-S318.

Harvill, E. T., Preston, A., Cotter, P. A., Allen, A. G., Maskell, D. J., & Miller, J. F. (2000) Multiple roles for *Bordetella* lipopolysaccharide molecules during respiratory tract infection. *Infection and Immunity.* **68** 6720-6728.

Hausman, S. Z., Manclark, C. R., & Burns, D. L. (1990) Binding of ATP by pertussis toxin and isolated toxin subunits. *Biochemistry.* **29**: 6128-6131.

Hausman, S. Z. & Burns, D. L. (1992) Interaction of pertussis toxin with cells and model membranes. *Journal of Biological Chemistry.* **267**: 13735-13739.

Hausman, S. Z. & Burns, D. L. (1993) Binding of pertussis toxin to lipid vesicles containing glycolipids. *Infection and Immunity.* **61**: 335-337.

Hayduk, E. J., Choe, L. H., & Lee, K. H. (2004) A two-dimensional electrophoresis map of Chinese hamster ovary cell proteins based on fluorescence staining. *Electrophoresis*. **25**: 2545-2556.

Hazenbos, W. L., Geuijen, C. A., van den Berg, B. M., Mooi, F. R., & van Furth, R. (1995) *Bordetella pertussis* fimbriae bind to human monocytes via the minor fimbrial subunit FimD. *Journal of Infectious Diseases*. **171**: 924-929.

Hazes, B., Boodhoo, A., Cockle, S. A., & Read, R. J. (1996) Crystal structure of the pertussis toxin-ATP complex: a molecular sensor. *Journal of Molecular Biology*. **258**: 661-671.

Hazes, B. & Read, R. J. (1997) Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. *Biochemistry*. **36**: 11051-11054.

Heerze, L. D. & Armstrong, G. D. (1990) Comparison of the lectin-like activity of pertussis toxin with two plant lectins that have differential specificities for alpha (2-6) and alpha (2-3)-linked sialic acid. *Biochemical and Biophysical Research Communication*. **172**: 1224-1229.

Heerze, L. D., Clark, C. G., Chen, Y., Smith, R. H., & Armstrong, G. D. (1991) Synthesis and characterization of a Pertussis toxin-biotin conjugate. *Biochemical and Biophysical Research Communication*. **179**: 1464-1469.

Heinemann writing group (2002) Edexcel GCSE mathematics higher course, In: Pledger, K., Sylvester, J., Medlow, C (Eds.) Heinemann educational publishers. Oxford, UK.

Heiss, L. N., Moser, S. A., Unanue, E. R., & Goldman, W. E. (1993) Interleukin-1 is linked to the respiratory epithelial cytopathology of pertussis. *Infection and Immunity*. **61**: 3123-3128.

Hellwig, S. M., Hazenbos, W. L., van de Winkel, J. G., & Mooi, F. R. (1999) Evidence for an intracellular niche for *Bordetella pertussis* in broncho-alveolar lavage cells of mice. *FEMS Immunology and Medical Microbiology*. **26**: 203-207.

Henderson, I. R. & Nataro, J. P. (2001) Virulence functions of autotransporter proteins. *Infection and Immunity*. **69**: 1231-1243.

Henningsen, R., Gale, B. L., Straub, K. M., & DeNagel, D. C. (2002) Application of zwitterionic detergents to the solubilization of integral membrane proteins for two-dimensional gel electrophoresis and mass spectrometry. *Proteomics*. **2**: 1479-1488.

Hewlett, E. L., Sauer, K. T., Myers, G. A., Cowell, J. L., & Guerrant, R. L. (1983) Induction of a novel morphological response in Chinese hamster ovary cells by pertussis toxin. *Infection and Immunity*. **40**:1198-1203.

Hodge, G., Hodge, S., Markus, C., Lawrence, A., & Han, P. (2003) A marked decrease in L-selectin expression by leucocytes in infants with *Bordetella pertussis* infection: leucocytosis explained? *Respirology*. **8**: 157-162.

Horiguchi, Y., Nakai, T., & Kume, K. (1991) Effects of *Bordetella bronchiseptica* dermonecrotic toxin on the structure and function of osteoblastic clone MC3T3-e1 cells. *Infection and Immunity.* **59**: 1112-1116.

Horiguchi, Y., Sugimoto, N., & Matsuda, M. (1993) Stimulation of DNA synthesis in osteoblast-like MC3T3-E1 cells by *Bordetella bronchiseptica* dermonecrotic toxin. *Infection and Immunity.* **61**: 3611-3615.

Horiguchi, Y., Senda, T., Sugimoto, N., Katahira, J., & Matsuda, M. (1995) *Bordetella bronchiseptica* dermonecrotizing toxin stimulates assembly of actin stress fibers and focal adhesions by modifying the small GTP-binding protein rho. *Journal of Cell Science.* **108**: 3243-3251.

Horiguchi, Y., Inoue, N., Masuda, M., Kashimoto, T., Katahira, J., Sugimoto, N., & Matsuda, M. (1997) *Bordetella bronchiseptica* dermonecrotizing toxin induces reorganization of actin stress fibers through deamidation of Gln-63 of the GTP-binding protein Rho. *Proceedings of the National Academy of the Sciences of the USA.* **94**: 11623-11626.

Horiuchi, Y., Takahashi, M., Asada, S., & Ishida, S. (1994) Increased levels of active pertussis toxin may aid a pertussis vaccine to pass the mouse body weight gain test *Biologicals.* **22**: 243-248.

Horiuchi, Y., Takahashi, M., Konda, T., Ochiai, M., Yamamoto, A., Kataoka, M., Toyoizumi, H., & Arakawa, Y. (2001) Quality control of diphtheria tetanus acellular pertussis combined (DTaP) vaccines in Japan *Japanese Journal of Infectious Diseases.* **54**: 167-180.

Hoving, S., Gerrits, B., Voshol, H., Muller, D., Roberts, R. C., & van Oostrum, J. (2002) Preparative two-dimensional gel electrophoresis at alkaline pH using narrow range immobilized pH gradients. *Proteomics.* **2**: 127-134.

Hudnall, S. D. & Molina, C. P. (2000) Marked increase in L-selectin-negative T cells in neonatal pertussis. The lymphocytosis explained? *American Journal of Clinical Pathology.* **114**: 35-40.

Ibsen, P. H. (1996) The effect of formaldehyde, hydrogen peroxide and genetic detoxification of pertussis toxin on epitope recognition by murine monoclonal antibodies. *Vaccine.* **14**: 359-368.

Irons, L. I., Ashworth, L. A., & Robinson, A. (1985) Release and purification of fimbriae from *Bordetella pertussis. Developents in Biological Standardization.* **61**: 153-163.

Ishibashi, Y., Claus, S., & Relman, D. A. (1994) *Bordetella pertussis* filamentous hemagglutinin interacts with a leukocyte signal transduction complex and stimulates bacterial adherence to monocyte CR3 (CD11b/CD18). *Journal of Experimental Medicine.* **180**: 1225-1233.

Ishibashi, Y., Yoshimura, K., Nishikawa, A., Claus, S., Laudanna, C., & Relman, D. A. (2002) Role of phosphatidylinositol 3-kinase in the binding of *Bordetella pertussis* to human monocytes. *Cellular Microbiology.* **4**, 825-833.

Ishida, S. (1968) Characterization of the body weight-decreasing toxicities in mice by the lymphocytosis-promoting factor and the heat-labile toxin of *B. pertussis* and endotoxin *Japanese Journal of Medical Science and Biology.* **21**: 115-135.

Islam, M. K., Miyoshi, T., Kasuga-Aoki, H., Isobe, T., Arakawa, T., Matsumoto, Y., & Tsuji, N. (2003) Inorganic pyrophosphatase in the roundworm Ascaris and its role in the development and molting process of the larval stage parasites. *European Journal of Biochemistry.* **270**: 2814-2826.

Jacob-Dubuisson, F., El Hamel, C., Saint, N., Guedin, S., Willery, E., Molle, G., & Locht, C. (1999) Channel formation by FhaC, the outer membrane protein involved in the secretion of the *Bordetella pertussis* filamentous hemagglutinin. *Journal of Biological Chemistry.* **274**: 37731-37735.

Jacob-Dubuisson, F. (2001) Two-partner secretion in Gram-negative bacteria: a thrifty, specific pathway for large virulence proteins. *Molecular Microbiology.* **40**: 306-313.

Janshoff, A., Steinem, C., Sieber, M., el Baya, A., Schmidt, M. A., & Galla, H. J. (1997) Quartz crystal microbalance investigation of the interaction of bacterial toxins with ganglioside containing solid supported membranes. *European Biophysics Journal.* vol. **26**: 261-270.

Jiang, W. & Schwendeman, S. P. (2000) Formaldehyde-mediated aggregation of protein antigens: comparison of untreated and formalinized model antigens. *Biotechnology and Bioengineering.* **70**: 507-517.

Jin, D. Y., Chae, H. Z., Rhee, S. G., & Jeang, K. T. (1997) Regulatory role for a novel human thioredoxin peroxidase in NF-kappaB activation. *Journal of Biological Chemistry.* **272**: 30952-30961.

Joao, H. C. & Dwek, R. A. (1993) Effects of glycosylation on protein structure and dynamics in ribonuclease B and some of its individual glycoforms. *European Journal of Biochemistry.* **218**: 239-244.

Kalchman, M. A., Graham, R. K., Xia, G., Koide, H. B., Hodgson, J. G., Graham, K. C., Goldberg, Y. P., Gietz, R. D., Pickart, C. M., & Hayden, M. R. (1996) Huntingtin is ubiquitinated and interacts with a specific ubiquitin-conjugating enzyme. *Journal of Biological Chemistry.* **271**: 19385-19394.

Kashimoto, T., Katahira, J., Cornejo, W. R., Masuda, M., Fukuoh, A., Matsuzawa, T., Ohnishi, T., & Horiguchi, Y. (1999) Identification of functional domains of *Bordetella* dermonecrotizing toxin. *Infection and Immunity.* **67**: 3727-3732.

Kaslow, H. R. & Burns, D. L. (1992) Pertussis toxin and target eukaryotic cells: binding, entry, and activation. *The FASEB Journal.* **6**: 2684-2690.

Katada, T. & Ui, M. (1976) Accelerated turnover of blood glucose in pertussis-sensitized rats due to combined actions of endogenous insulin and adrenergic beta-stimulation. *Biochimica et Biophysica Acta.* **421**: 57-69.

Katada, T. & Ui, M. (1977) Perfusion of the pancreas isolated from pertussis-sensitized rats: potentiation of insulin secretory responses due to beta-adrenergic stimulation *Endocrinology.* **101**: 1247-1255.

Katada, T. & Ui, M. (1979) Effect of in vivo pretreatment of rats with a new protein purified from *Bordetella pertussis* on in vitro secretion of insulin: role of calcium. *Endocrinology.* **104**: 1822-1827.

Kataoka, M., Toyoizumi, H., Yamamoto, A., Ochiai, M., & Horiuchi, Y. (2002) Chinese hamster ovary (CHO) cell clustering does not correlate with in vivo histamine-sensitization when measuring residual activity of aldehyde-treated pertussis toxin (PT). *Biologicals.* **30**: 297-302.

Katz, J. B., Hanson, S. K., Patterson, P. A., & Stoll, I. R. (1989) In vitro assessment of viral antigen content in inactivated aluminum hydroxide adjuvanted vaccines *Journal of Virological Methods.* **25**: 101-108.

Kerfoot, S. M., Long, E. M., Hickey, M. J., Andonegui, G., Lapointe, B. M., Zanardo, R. C., Bonder, C., James, W. G., Robbins, S. M., & Kubes, P. (2004) TLR4 contributes to disease-inducing mechanisms resulting in central nervous system autoimmune disease. *Journal of Immunology.* **173**: 7070-7077.

Khelef, N., Zychlinsky, A., & Guiso, N. (1993) *Bordetella pertussis* induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. *Infection and Immunity.* **61**: 4064-4071.

Kikuchi, J., Furukawa, Y., Kubo, N., Tokura, A., Hayashi, N., Nakamura, M., Matsuda, M., & Sakurabayashi, I. (2000) Induction of ubiquitin-conjugating enzyme by aggregated low density lipoprotein in human macrophages and its implications for atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **20**:128-134.

Kirsch, T., Harrison, G., Golub, E. E., & Nah, H. D. (2000) The roles of annexins and types II and X collagen in matrix vesicle-mediated mineralization of growth plate cartilage. *Journal of Biological Chemistry.* **275**: 35577-35583.

Klein, D. L. (1995) Multicenter acellular pertussis vaccine trial: a National Institutes of Health perspective. *Pediatrics.* **96**: 547-548.

Ko, K. S., Peck, K. R., Oh, W. S., Lee, N. Y., Lee, J. H., & Song, J. H. (2005) New species of *Bordetella, Bordetella ansorpii* sp. nov., isolated from the purulent exudate of an epidermal cyst. *Journal of Clinical Microbiology.* **43**: 2516-2519.

Kolb, J. P., Genot, E., Petit-Koskas, E., Paul-Eugene, N., & Dugas, B. (1990) Effect of bacterial toxins on human B cell activation. I. Mitogenic activity of pertussis toxin *European Journal of Immunology.* **20**: 969-976.

Kotob, S. I., Hausman, S. Z., & Burns, D. L. (1995) Localization of the promoter for the ptl genes of *Bordetella pertussis*, which encode proteins essential for secretion of pertussis toxin. *Infection and Immunity.* **63**: 3227-3230.

Krueger, K. M. & Barbieri, J. T. (1995) The family of bacterial ADP-ribosylating exotoxins. *Clinical Microbiology Reviews.* **8**: 34-47.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**: 680-685.

Lambert-Buisine, C., Willery, E., Locht, C., & Jacob-Dubuisson, F. (1998) N-terminal characterization of the *Bordetella pertussis* filamentous haemagglutinin. *Molecular Microbiology.* **28**: 1283-1293.

Lang, A. B., Ganss, M. T., & Cryz, S. J., Jr. (1989) Monoclonal antibodies that define neutralizing epitopes of pertussis toxin: conformational dependence and epitope mapping. *Infection and Immunity.* **57**: 2660-2665.

Lapin J.H. (1943) Whooping Cough, Thomas, Springfield.

Lee, M. S., Kim, K. W., Kim, Y. H., & Lee, G. M. (2003) Proteome analysis of antibody-expressing CHO cells in response to hyperosmotic pressure. *Biotechnology Progress.* **19**: 1734-1741.

Leffers, H., Nielsen, M. S., Andersen, A. H., Honore, B., Madsen, P., Vandekerckhove, J., & Celis, J. E. (1993) Identification of two human Rho GDP dissociation inhibitor proteins whose overexpression leads to disruption of the actin cytoskeleton. *Experimental Cell Research.* **209**: 165-174.

Lim, L. K., Sekura, R. D., & Kaslow, H. R. (1985) Adenine nucleotides directly stimulate pertussis toxin. *Journal of Biological Chemistry.* **260**: 2585-2588.

Livey, I. & Wardlaw, A. C. (1984) Production and properties of *Bordetella pertussis* heat-labile toxin. *Journal of Medical Microbiology.* **17**: 91-103.

Llorca, O., Martin-Benito, J., Ritco-Vonsovici, M., Grantham, J., Hynes, G. M., Willison, K. R., Carrascosa, J. L., & Valpuesta, J. M. (2000) Eukaryotic chaperonin CCT stabilizes actin and tubulin folding intermediates in open quasi-native conformations. *EMBO Journal.* **19**: 5971-5979.

Lobet, Y., Feron, C., Dequesne, G., Simoen, E., Hauser, P., & Locht, C. (1993) Site-specific alterations in the B oligomer that affect receptor-binding activities and mitogenicity of pertussis toxin. *The Journal of Experimental Medicine.* **177**: 79-87.

Loscher, C.E., Donnelly, S., Mills, K.H., Lynch, M.A. (2000) Interleukin-1beta-dependent changes in the hippocampus following parenteral immunization with a whole cell pertussis vaccine. *Journal of Neuroimmunology.* **111**: 68-76

Locht, C. & Keith, J. M. (1986) Pertussis toxin gene: nucleotide sequence and genetic organization. *Science.* **232**: 1258-1264.

Locht, C. (1999). Molecular aspects of *Bordetella Pertussis* Pathogenesis. *International Microbiology*. **2**: 301:305.

Loosmore, S., Zealey, G., Cockle, S., Boux, H., Chong, P., Yacoob, R., & Klein, M. (1993) Characterization of pertussis toxin analogs containing mutations in B-oligomer subunits. *Infection and Immunity*. **61**: 2316-2324.

Loosmore, S. M., Zealey, G. R., Boux, H. A., Cockle, S. A., Radika, K., Fahim, R. E., Zobrist, G. J., Yacoob, R. K., Chong, P. C., Yao, F. L., *et al.* (1990) Engineering of genetically detoxified pertussis toxin analogs for development of a recombinant whooping cough vaccine. *Infection and Immunity*. **58**: 3653-3662.

Luduena, R. F. & Roach, M. C. (1991) Tubulin sulfhydryl groups as probes and targets for antimitotic and antimicrotubule agents. *Pharmacology and Therapeutics*. **49**: 133-152.

Ma, R. Z., Gao, J., Meeker, N. D., Fillmore, P. D., Tung, K. S., Watanabe, T., Zachary, J. F., Offner, H., Blankenhorn, E. P., & Teuscher, C. (2002) Identification of Bphs, an autoimmune disease locus, as histamine receptor H1. *Science*. **297**: 620-623.

Macdonald-Fyall, J., Xing, D., Corbel, M., Baillie, S., Parton, R., & Coote, J. (2004) Adjuvanticity of native and detoxified adenylate cyclase toxin of *Bordetella pertussis* towards co-administered antigens. *Vaccine*. **22**: 4270-4281.

Marouga, R., David, S., & Hawkins, E. (2005) The development of the DIGE system: 2D fluorescence difference gel analysis technology. *Analytical and Bioanalytical Chemistry*. **382**: 669-678.

Martin, C., Requero, M. A., Masin, J., Konopasek, I., Goni, F. M., Sebo, P., & Ostolaza, H. (2004) Membrane restructuring by *Bordetella pertussis* adenylate cyclase toxin, a member of the RTX toxin family. *Journal of Bacteriology*. **186**: 3760-3765.

Mastrantonio, P., Cerquetti, M., Cardines, R., Lande, R., Ausiello, C. M., & Cassone, A. (1999) Immunogenicity issues in the quality control of the new acellular pertussis vaccines. *Biologicals*. **27**: 119-121.

Masuda, M., Minami, M., Shime, H., Matsuzawa, T., & Horiguchi, Y. (2002) In vivo modifications of small GTPase Rac and Cdc42 by *Bordetella* dermonecrotic toxin. *Infection and Immunity*. **70**: 998-1001.

Matsuzawa, T., Kashimoto, T., Katahira, J., & Horiguchi, Y. (2002) Identification of a receptor-binding domain of *Bordetella* dermonecrotic toxin. *Infection and Immunity*. **70**: 3427-3432.

Matsuzawa, T., Fukui, A., Kashimoto, T., Nagao, K., Oka, K., Miyake, M., & Horiguchi, Y. (2004) *Bordetella* dermonecrotic toxin undergoes proteolytic processing to be translocated from a dynamin-related endosome into the cytoplasm in an acidification-independent manner. *Journal of Biological Chemistry*. **279**: 2866-2872.

Mattoo, S. & Cherry, J. D. (2005) Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other Bordetella subspecies. *Clinical Microbiology Reviews.* **18**: 326-382.

Mawas, F., Newman, G., Burns, S., & Corbel, M. J. (2005) Suppression and modulation of cellular and humoral immune responses to *Haemophilus influenzae* type b (Hib) conjugate vaccine in hib-diphtheria-tetanus toxoids-acellular pertussis combination vaccines: a study in a rat model. *Journal of Infectious Diseases.* **191**: 58-64.

Mawas, F., Dickinson, R., Douglas-Bardsley, A., Xing, D. K., Sesardic, D., & Corbel, M. J. (2006) Immune interaction between components of acellular pertussis-diphtheria-tetanus (DTaP) vaccine and *Haemophilus influenzae* b (Hib) conjugate vaccine in a rat model. *Vaccine.* **24**: 3505-3512.

McIntosh, D. B. (1992) Glutaraldehyde cross-links Lys-492 and Arg-678 at the active site of sarcoplasmic reticulum Ca(2+)-ATPase *Journal of Biological Chemistry.* **267**: 22328-22335.

McVernon, J., Andrews, N., Slack, M. P., & Ramsay, M. E. (2003) Risk of vaccine failure after Haemophilus influenzae type b (Hib) combination vaccines with acellular pertussis. *Lancet.* **361**: 1521-1523.

Means, G. E. & Feeney, R. E. (1971) Affinity labeling of pancreatic ribonuclease. *Journal of Biological Chemistry.* **246**: 5532-5533.

Medical Research Council (1956) Vaccination against whooping-cough: relation between protection in children and results of laboratory tests. *British Medical Journal* **2**: 454-462.

Menozzi, F. D., Gantiez, C., & Locht, C. (1991) Interaction of the *Bordetella pertussis* filamentous hemagglutinin with heparin. *FEMS Microbiology Letters.* **62**: 59-64.

Menozzi, F. D., Debrie, A. S., Tissier, J. P., Locht, C., Pethe, K., & Raze, D. (2002) Interaction of human Tamm-Horsfall glycoprotein with *Bordetella pertussis* toxin. *Microbiology.* **148**: 1193-1201.

Metz, B., Kersten, G. F., Hoogerhout, P., Brugghe, H. F., Timmermans, H. A., de Jong, A., Meiring, H., ten Hove, J., Hennink, W. E., Crommelin, D. J., & Jiskoot, W. (2004) Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. *Journal of Biological Chemistry.* **279**: 6235-6243.

Mierzwa, S. & Chan, S. K. (1987) Chemical modification of human alpha 1-proteinase inhibitor by tetranitromethane. Structure-function relationship. *Biochemical Journal.* **246**: 37-42.

Miller, D. L., Ross, E. M., Alderslade, R., Bellman, M. H., & Rawson, N. S. (1981) Pertussis immunisation and serious acute neurological illness in children. *British Medical Journal (Clinical research ed.).* **282**: 1595-1599.

Mills, K. H. & Redhead, K. (1993) Cellular immunity in pertussis. *Journal of Medical Microbiology.* **39**: 163-164.

Mills, K. H., Brady, M., Ryan, E., & Mahon, B. P. (1998a) A respiratory challenge model for infection with *Bordetella pertussis*: application in the assessment of pertussis vaccine potency and in defining the mechanism of protective immunity. *Developments in Biological Standardization.* **95**: 31-41.

Mills, K. H., Ryan, M., Ryan, E., & Mahon, B. P. (1998b) A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. *Infection and Immunity.* **66**: 594-602.

Mobberley-Schuman, P. S. & Weiss, A. A. (2005) Influence of CR3 (CD11b/CD18) expression on phagocytosis of *Bordetella pertussis* by human neutrophils. *Infection and Immunity.* **73**: 7317-7323.

Mollenhauer, J. (1997) Annexins: what are they good for? *Cellular and Molecular Life Sciences.* **53**: 506-507.

Molloy, M. P. (2000) Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. *Analytical Biochemistry.* **280**: 1-10.

Mooi, F. R., He, Q., van Oirschot, H., & Mertsola, J. (1999) Variation in the *Bordetella pertussis* virulence factors pertussis toxin and pertactin in vaccine strains and clinical isolates in Finland. *Infection and Immunity.* **67**: 3133-3134.

Mooi, F. R., van Loo, I. H., & King, A. J. (2001) Adaptation of *Bordetella pertussis* to vaccination: a cause for its reemergence? *Emerging Infectious Diseases.* **7**: 526-528.

Morrison, E. E., Wardleworth, B. N., Askham, J. M., Markham, A. F., & Meredith, D. M. (1998) EB1, a protein which interacts with the APC tumour suppressor, is associated with the microtubule cytoskeleton throughout the cell cycle. *Oncogene.* **17**: 3471-3477.

Morse, S. I. & Riester, S. K. (1967) Studies on the leukocytosis and lymphocytosis induced by Bordetella pertussis. I. Radioautographic analysis of the circulating cells in mice undergoing pertussis-induced hyperleukocytosis. *The Journal of Experimental Medicine.* **125**: 401-408.

Morse, S. I. & Morse, J. H. (1976) Isolation and properties of the leukocytosis- and lymphocytosis-promoting factor of *Bordetella pertussis*. *The Journal of Experimental Medicine.* **143**: 1483-1502.

Munoz, J. J. & Peacock, M. G. (1989) Role of pertussigen (pertussis toxin) on the mouse protective activity of vaccines made from *Bordetella* species. *Microbiology and Immunology.* **33**: 341-355.

Munoz, J. J. & Peacock, M. G. (1990) Action of pertussigen (pertussis toxin) on serum IgE and on Fc epsilon receptors on lymphocytes. *Cellular Immunology.* **127**: 327-336.

Munoz, J.J. (1985) Biological activities of pertussigen (pertussis toxin), pg 327-337 In: R.D. Sekura, J. Moss, M. Vaughan (Eds.), Pertussis Toxin. Academic press inc, London, England 1985, pp. 1-18.

Munoz, J & Bergman, R.K. (1968) Histamine-sensitizing factors from microbial agents, with special reference to *Bordetella pertussis*. Bacteriological Reviews. **32**: 103-126.

Munro, P., Flatau, G., Anjuere, F., Hofman, V., Czerkinsky, C., & Lemichez, E. (2005) The Rho GTPase activators CNF1 and DNT bacterial toxins have mucosal adjuvant properties. *Vaccine*. **23**: 2551-2556.

Nakano, M., Kakehi, K., Tsai, M. H., & Lee, Y. C. (2004) Detailed structural features of glycan chains derived from alpha1-acid glycoproteins of several different animals: the presence of hypersialylated, O-acetylated sialic acids but not disialyl residues. *Glycobiology*. **14**: 431-441.

Naryzhny, S. N. & Lee, H. (2001) Protein profiles of the Chinese hamster ovary cells in the resting and proliferating stages. *Electrophoresis*. **22**: 1764-1775.

Nencioni, L., Pizza, M. G., Volpini, G., De Magistris, M. T., Giovannoni, F., & Rappuoli, R. (1991a) Properties of the B oligomer of pertussis toxin. *Infection and Immunity*. **59**: 4732-4734.

Nencioni, L., Volpini, G., Peppoloni, S., Bugnoli, M., De Magistris, T., Marsili, I., & Rappuoli, R. (1991b) Properties of pertussis toxin mutant PT-9K/129G after formaldehyde treatment. *Infection and Immunity*. **59**: 625-630.

Nicosia, A., Perugini, M., Franzini, C., Casagli, M. C., Borri, M. G., Antoni, G., Almoni, M., Neri, P., Ratti, G., & Rappuoli, R. (1986) Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication. *Proceedings of the National Academy of Sciences of the USA*. **83**: 4631-4635.

Nijtmans, L. G., De Jong, L., Artal, S. M., Coates, P. J., Berden, J. A., Back, J. W., Muijsers, A. O., van der, S. H., & Grivell, L. A. (2000) Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. *EMBO Journal*. **19**: 2444-2451.

Njamkepo, E., Pinot, F., Francois, D., Guiso, N., Polla, B. S., & Bachelet, M. (2000) Adaptive responses of human monocytes infected by *Bordetella pertussis*: the role of adenylate cyclase hemolysin. *Journal of Cellular Physiology*. **183**: 91-99.

Nogimori, K., Ito, K., Tamura, M., Satoh, S., Ishii, S., & Ui, M. (1984) Chemical modification of islet-activating protein, pertussis toxin. Essential role of free amino groups in its lymphocytosis-promoting activity. *Biochimica et Biophysica Acta*. **801**: 220-231.

Nogimori, K., Tamura, M., Yajima, M., Hashimura, N., Ishii, S., & Ui, M. (1986) Structure-function relationship of islet-activating protein, pertussis toxin: biological activities of hybrid toxins reconstituted from native and methylated subunits. *Biochemistry*. **25**: 1355-1363.

Northrup, R. S. & Chisari, F. V. (1972) Response of monkeys to immunization with cholera toxoid, toxin, and vaccine: reversion of cholera toxoid. *Journal of Infectious Diseases*. **125**: 471-479.

Ogino, T., Yamadera, T., Nonaka, T., Imajoh-Ohmi, S., & Mizumoto, K. (2001) Enolase, a cellular glycolytic enzyme, is required for efficient transcription of Sendai virus genome. *Biochemical and Biophysical Research Communication.* **285**: 447-455.

Okuyama, H., Nakamura, H., Shimahara, Y., Araya, S., Kawada, N., Yamaoka, Y., & Yodoi, J. (2003) Overexpression of thioredoxin prevents acute hepatitis caused by thioacetamide or lipopolysaccharide in mice. *Hepatology.* **37**: 1015-1025.

Olin, P., Rasmussen, F., Gustafsson, L., Hallander, H. O., & Heijbel, H. (1997) Randomised controlled trial of two-component, three-component, and five-component acellular pertussis vaccines compared with whole-cell pertussis vaccine. Ad Hoc Group for the Study of Pertussis Vaccines. *Lancet.* **350**: 1569-1577.

Olin, P., Hallander, H. O., Gustafsson, L., Reizenstein, E., & Storsaeter, J. (2001) How to make sense of pertussis immunogenicity data. *Clinical Infectious Diseases.* **33**: S288-S291.

Oliver, D. C., Huang, G., Nodel, E., Pleasance, S., & Fernandez, R. C. (2003) A conserved region within the Bordetella pertussis autotransporter BrkA is necessary for folding of its passenger domain. *Molecular Microbiology.* **47**: 1367-1383.

Oliver, D. C., Huang, G., & Fernandez, R. C. (2003) Identification of secretion determinants of the Bordetella pertussis BrkA autotransporter. *Journal of Bacteriology.* **185**: 489-495.

Parfentjev, I. A., and M. A. Goodline. (1948) Histamine shock in mice sensitized with *Hemophilus pertussis* vaccine. *The Journal of Pharmacology and Experimental Therapeutics.* **92**:411.

Parton, R. (1985) Effect of prednisolone on the toxicity of *Bordetella pertussis* for mice. *Journal of Medical Microbiology.* **19**: 391-400.

Parton, R. (1998). *Bordetella.* pg 901-908 In: Baslows, A. & Duerden B. (Eds.), Topley and Wilsons Microbiology and Microbial Infections. Arnold, London, England.

Patterson, C. E., Stasek, J. E., Schaphorst, K. L., Davis, H. W., & Garcia, J. G. (1995) Mechanisms of pertussis toxin-induced barrier dysfunction in bovine pulmonary artery endothelial cell monolayers. *The American Journal of Physiology.* **268**: L926-L934.

Petre, J., Pizza, M., Nencioni, L., Podda, A., De Magistris, M. T., & Rappuoli, R. (1996) The reaction of bacterial toxins with formaldehyde and its use for antigen stabilization. *Developments in Biological Standardization.* **87**: 125-134.

Pichichero, M. E., DeTora, L. M., & Johnson, D. R. (2006) An adolescent and adult formulation combined tetanus, diphtheria and five-component pertussis vaccine. *Expert Review of Vaccines.* **5**: 175-187.

Pieroni, R. E., Broderick, E. J., & Levine, L. (1966) Endotoxin-induced hypersensitivity to histamine in mice. I. Contrasting effects of bacterial lipopolysaccharides and the classical histamine-sensitizing factor of *Bordetella pertussis*. *Journal of Bacteriology.* **91**: 2169-2174.

Pizza, M., Bartoloni, A., Prugnola, A., Silvestri, S., & Rappuoli, R. (1988) Subunit S1 of pertussis toxin: mapping of the regions essential for ADP-ribosyltransferase activity. *Proceedings of the National Academy Sciences of the USA*. **85**: 7521-7525.

Pizza, M., Covacci, A., Bartoloni, A., Perugini, M., Nencioni, L., De Magistris, M. T., Villa, L., Nucci, D., Manetti, R., Bugnoli, M., *et al* . (1989) Mutants of pertussis toxin suitable for vaccine development. *Science*. **246**: 497-500.

Porter, K., Prescott, D., & Frye, J. (1973) Changes in surface morphology of Chinese hamster ovary cells during the cell cycle. *The Journal of Cell Biology*. **57**: 815-836.

Prasad, S. M., Yin, Y., Rodzinski, E., Tuomanen, E. I., & Masure, H. R. (1993) Identification of a carbohydrate recognition domain in filamentous hemagglutinin from *Bordetella pertussis*. *Infection and Immunity*. **61**: 2780-2785.

Preston, N.W., Timewell, R. M., & Carter, E.J. (1980) Experimental pertussis infection in the rabbit: similarities with infection in primates. *Journal of Infection*. **2**: 227-235

Preston, A., Parkhill, J., & Maskell, D. J. (2004) The bordetellae: lessons from genomics. *Nature reviews. Microbiology*. **2**: 379-390.

Preston, A. 2005, *Bordetella pertussis*: the intersection of genomics and pathobiology. *Canadian Medical Association Journal*. **173**: 55-62.

Preston, N. W. (1985) Essential immunogens in human pertussis: the role of fimbriae. *Developments in Biological Standardization*. **61**: 137-141.

Rabilloud, T. (1998) Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. *Electrophoresis*. **19**: 758-760.

Rappuoli, R., Pizza, M., Podda, A., De Magistris, M. T., Ceccarini, C., & Nencioni, L. (1994) Development of the new acellular recombinant pertussis vaccine. *Archives de l'Institut Pasteur de Tunis*. **71**: 557-563.

Ramagli, L.S & Rodriguez, L.V. (1985) Quantitation of microgram amounts of protein in two-dimensional gel electrophoresis sample buffer. *Electrophoresis*. **6**: 559-563

Rawitch, A. B., Pollock, H. G., & Yang, S. X. (1993) Thyroglobulin glycosylation: location and nature of the N-linked oligosaccharide units in bovine thyroglobulin. *Archives of Biochemistry and Biophysics*. **300**: 271-279.

Raze, D., Sato, H., Antoine, R., & Locht, C (1998) Genetic exchange of the S2 and S3 subunits in pertussis toxin, pg 66-67 In: Hacker et al, (Eds.), Bacterial protein toxins, Zent.bl.Bakteriol.Suppl.29. Gustav Fischer, Jena, Stuttgart, Lubeck, Ulm.

Relman, D., Tuomanen, E., Falkow, S., Golenbock, D. T., Saukkonen, K., & Wright, S. D. (1990) Recognition of a bacterial adhesion by an integrin: macrophage CR3 (alpha M beta 2, CD11b/CD18) binds filamentous hemagglutinin of Bordetella pertussis. *Cell*. **61**: 1375-1382.

Renieri, A., Meloni, I., Longo, I., Ariani, F., Mari, F., Pescucci, C., & Cambi, F. (2003) Rett syndrome: the complex nature of a monogenic disease. *Journal of Molecular Medicine.* **81**: 346-354.

Rennels, M. B., Deloria, M. A., Pichichero, M. E., Losonsky, G. A., Englund, J. A., Meade, B. D., Anderson, E. L., Steinhoff, M. C., & Edwards, K. M. (2000) Extensive swelling after booster doses of acellular pertussis-tetanus-diphtheria vaccines. *Pediatrics.* **105**: e12.

Riebe, D. & Thorn, W. (1991) Influence of carbohydrate moieties of human serum transferrin on the determination of its molecular mass by polyacrylamide gradient gel electrophoresis and staining with periodic acid-Schiff reagent. *Electrophoresis.* **12**: 287-293.

Robbinson, D., Cockle, S., Singh, B., & Strejan, G. H. (1996) Native, but not genetically inactivated, pertussis toxin protects mice against experimental allergic encephalomyelitis. *Cellular Immunology.* **168**: 165-173.

Roberts, M., Bacon, A., Rappuoli, R., Pizza, M., Cropley, I., Douce, G., Dougan, G., Marinaro, M., McGhee, J., & Chatfield, S. (1995) A mutant pertussis toxin molecule that lacks ADP-ribosyltransferase activity, PT-9K/129G, is an effective mucosal adjuvant for intranasally delivered proteins. *Infection and Immunity.* **63**: 2100-2108.

Robinson, A. & Irons, L. I. (1983) Synergistic effect of *Bordetella pertussis* lymphocytosis-promoting factor on protective activities of isolated *Bordetella* antigens in mice. *Infection and Immunity.* **40**: 523-528.

Rodriguez, M. E., Hellwig, S. M., Perez Vidakovics, M. L., Berbers, G. A., & van de Winkel, J. G. (2006) *Bordetella pertussis* attachment to respiratory epithelial cells can be impaired by fimbriae-specific antibodies. *FEMS Immunology and Medical Microbiology.* **46**: 39-47.

Rogers, S. L., Rogers, G. C., Sharp, D. J., & Vale, R. D. (2002) Drosophila EB1 is important for proper assembly, dynamics, and positioning of the mitotic spindle. *The Journal of Cell Biology.* **158**: 873-884.

Rogers, T. S., Corey, S. J., & Rosoff, P. M. (1990) Identification of a 43-kilodalton human T lymphocyte membrane protein as a receptor for pertussis toxin. *Journal of Immunology.* **145**: 678-683.

Rosenthal, R. S., Nogami, W., Cookson, B. T., Goldman, W. E., & Folkening, W. J. (1987) Major fragment of soluble peptidoglycan released from growing *Bordetella pertussis* is tracheal cytotoxin. *Infection and Immunity.* **55**: 2117-2120.

Rosoff, P. M., Walker, R., & Winberry, L. (1987) Pertussis toxin triggers rapid second messenger production in human T lymphocytes. *Journal of Immunology.* **139**: 2419-2423.

Rudd, P. M., Woods, R. J., Wormald, M. R., Opdenakker, G., Downing, A. K., Campbell, I. D., & Dwek, R. A. (1995) The effects of variable glycosylation on the functional activities of ribonuclease, plasminogen and tissue plasminogen activator. *Biochimica et Biophysica Acta.* **1248**: 1-10.

Rutishauser, J. & Spiess, M. (2002) Endoplasmic reticulum storage diseases. *Swiss Medical Weekly.* **132**: 211-222.

Ryan, M., McCarthy, L., Rappuoli, R., Mahon, B. P., & Mills, K. H. (1998) Pertussis toxin potentiates Th1 and Th2 responses to co-injected antigen: adjuvant action is associated with enhanced regulatory cytokine production and expression of the co-stimulatory molecules B7-1, B7-2 and CD28. *International Immunology.* **10**: 651-662.

Sanchez, J. C., Rouge, V., Pisteur, M., Ravier, F., Tonella, L., Moosmayer, M., Wilkins, M. R., & Hochstrasser, D. F. (1997) Improved and simplified in-gel sample application using reswelling of dry immobilized pH gradients. *Electrophoresis.* **18**: 324-327.

Sato, H. & Sato, Y. (1984) *Bordetella pertussis* infection in mice: correlation of specific antibodies against two antigens, pertussis toxin, and filamentous haemagglutinin with mouse protectivity in an intracerebral or aerosol challenge system. *Infection and Immunity.* **46**: 415-421.

Sato, H. & Sato, Y. (1985) Protective antigens of *Bordetella pertussis* mouse-protection test against intracerebral and aerosol challenge of *B. pertussis. Developments in Biological Standardization.* **61**: 461-467.

Sato, Y., Arai, H., & Suzuki, K. (1974) Leukocytosis-promoting factor of *Bordetella pertussis*. 3. Its identity with protective antigen. *Infection and Immunity.* **9**: 801-810.

Sato, Y., Cowell, J. L., Sato, H., Burstyn, D. G., & Manclark, C. R. (1983) Separation and purification of the hemagglutinins from *Bordetella pertussis. Infection and Immunity.* **41**: 313-320.

Sato, Y., Kimura, M., & Fukumi, H. (1984) Development of a pertussis component vaccine in Japan. *Lancet.* **1**: 122-126.

Sato, Y. & Sato, H. (1988) Further characterization of Japanese acellular pertussis vaccine prepared in 1988 by 6 Japanese manufacturers. *The Tokai Journal of Experimental and Clinical Medicine.* **13**: 79-88.

Saukkonen, K., Burnette, W. N., Mar, V. L., Masure, H. R., & Tuomanen, E. I. (1992) Pertussis toxin has eukaryotic-like carbohydrate recognition domains. *Proceedings of the National Academy Sciences of the USA.* **89**: 118-122.

Seong, J.K., Kim Do, K., Choi, K.H., Oh, S.H., Lee, S-S., Um, H-D (2002) Proteomic analysis of the cellular proteins induced by adaptive concentrations of hydrogen peroxide in human U937 cells. Experimental and Molecular Medicine. **34**: 374-378

Scapigliati, G., Rappuoli, R., Silvestri, S., & Pallini, V. (1988) Cytoskeletal alterations as a parameter for assessment of toxicity. *Xenobiotica.* **18**: 715-724.

Schaeffer, L. M., McCormack, F. X., Wu, H., & Weiss, A. A. (2004) Interactions of pulmonary collectins with *Bordetella bronchiseptica* and *Bordetella pertussis* lipopolysaccharide elucidate the structural basis of their antimicrobial activities *Infection and Immunity.* **72**: 7124-7130.

Scheler, C., Lamer, S., Pan, Z., Li, X. P., Salnikow, J., & Jungblut, P. (1998) Peptide mass fingerprint sequence coverage from differently stained proteins on two-dimensional electrophoresis patterns by matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS). *Electrophoresis.* **19**: 918-927.

Schmidt, G., Goehring, U. M., Schirmer, J., Lerm, M., & Aktories, K. (1999) Identification of the C-terminal part of *Bordetella* dermonecrotic toxin as a transglutaminase for rho GTPases. *Journal of Biological Chemistry.* **274**: 31875-31881.

Schmidt, M. A. & Schmidt, W. (1989) Inhibition of pertussis toxin binding to model receptors by antipeptide antibodies directed at an antigenic domain of the S2 subunit *Infection and Immunity.* **57**: 3828-3833.

Schmidt, M. A., Raupach, B., Szulczynski, M., & Marzillier, J. (1991) Identification of linear B-cell determinants of pertussis toxin associated with the receptor recognition site of the S3 subunit. *Infection and Immunity.* **59**: 1402-1408.

Schwarz, K., Eggers, M., Soza, A., Koszinowski, U. H., Kloetzel, P. M., & Groettrup, M. (2000) The proteasome regulator PA28alpha/beta can enhance antigen presentation without affecting 20S proteasome subunit composition. *European Journal of Immunology.* **30**: 3672-3679.

Sekura, R. D., Fish, F., Manclark, C. R., Meade, B., & Zhang, Y. L. (1983) Pertussis toxin. Affinity purification of a new ADP-ribosyltransferase. *Journal of Biological Chemistry.* **258**: 14647-14651.

Sekura, R.D., Zhang, Y-L., Quetin-Millet, M.J. (1985). Pertussis toxin: structural elements involved in the interaction with cells, pg 1-18 in: R.D. Sekura, J. Moss, M. Vaughan, Pertussis toxin. Academic press inc, London, England.

Shapiro, M. D., Hanken, J., & Rosenthal, N. (2003) Developmental basis of evolutionary digit loss in the Australian lizard Hemiergis. *Journal of Experimental Zoology. Part B. Molecular and Developmental Evolution.* **297**: 48-56.

Sharp, D. J., Rogers, G. C., & Scholey, J. M. (2000) Microtubule motors in mitosis. *Nature.* **407**: 41-47.

Shaw, M. M. & Riederer, B. M. (2003) Sample preparation for two-dimensional gel electrophoresis. *Proteomics.* **3**: 1408-1417.

Sheu, G. T. & Traugh, J. A. (1997) Recombinant subunits of mammalian elongation factor 1 expressed in *Escherichia coli*. Subunit interactions, elongation activity, and phosphorylation by protein kinase CKII. *Journal of Biological Chemistry.* **272**: 33290-33297.

Siber, G. R., Thakrar, N., Yancey, B. A., Herzog, L., Todd, C., Cohen, N., Sekura, R. D., & Lowe, C. U. (1991) Safety and immunogenicity of hydrogen peroxide-inactivated pertussis toxoid in 18-month-old children. *Vaccine.* **9**: 735-740.

Simondon, F., Preziosi, M. P., Yam, A., Kane, C. T., Chabirand, L., Iteman, I., Sanden, G., Mboup, S., Hoffenbach, A., Knudsen, K., Guiso, N., Wassilak, S., & Cadoz, M. (1997) A randomized double-blind trial comparing a two-component acellular to a whole-cell pertussis vaccine in Senegal. *Vaccine.* **15**: 1606-1612.

Sindt, K. A., Hewlett, E. L., Redpath, G. T., Rappuoli, R., Gray, L. S., & Vandenberg, S. R. (1994) Pertussis toxin activates platelets through an interaction with platelet glycoprotein Ib. *Infection and Immunity.* **62**: 3108-3114.

Singh, B., Soltys, B. J., Wu, Z. C., Patel, H. V., Freeman, K. B., & Gupta, R. S. (1997) Cloning and some novel characteristics of mitochondrial Hsp70 from Chinese hamster cells. *Experimental Cell Research.* **234**: 205-216.

Sokolovsky, M., Riordan, J. F., & Vallee, B. L. (1966) Tetranitromethane. A reagent for the nitration of tyrosyl residues in proteins. *Biochemistry.* **5**: 3582-3589.

Spangler, B. D., Heerze, L. D., Clark, C. G., & Armstrong, G. D. (1993) Hydrophobic binding of pertussis toxin is enhanced by oligosaccharide receptors. *Archives of Biochemistry and Biophysics.* **305**: 153-158.

Spangrude, G. J., Braaten, B. A., & Daynes, R. A. (1984) Molecular mechanisms of lymphocyte extravasation. I. Studies of two selective inhibitors of lymphocyte recirculation. *Journal of Immunology.* **132**: 354-362.

Spear, E. D. & Ng, D. T. (2003) Stress tolerance of misfolded carboxypeptidase Y requires maintenance of protein trafficking and degradative pathways. *Molecular Biology of the Cell.* **14**: 2756-2767.

Spik, G., Bayard, B., Fournet, B., Strecker, G., Bouquelet, S., & Montreuil, J. (1975) Studies on glycoconjugates. LXIV. Complete structure of two carbohydrate units of human serotransferrin. *FEBS Letters.* **50**: 296-299.

Spiro, R. G. (1960) Studies on fetuin, a glycoprotein of fetal serum. I. Isolation, chemical composition, and physiochemical properties. *Journal of Biological Chemistry.* **235**: 2860-2869.

Spiro, R. G. & Spiro, M. J. (1965) The carbohydrate composition of the thyroglobulins from several species. *Journal of Biological Chemistry.* **240**: 997-1001.

Stanley, J. B., Gorczynski, R., Huang, C. K., Love, J., & Mills, G. B. (1990) Tyrosine phosphorylation is an obligatory event in IL-2 secretion. *Journal of Immunology.* **145**: 2189-2198.

Stathopoulos, C., Hendrixson, D. R., Thanassi, D. G., Hultgren, S. J., St, G. J., III, & Curtiss, R., III (2000) Secretion of virulence determinants by the general secretory pathway in gram-negative pathogens: an evolving story. *Microbes and Infection.* **2**: 1061-1072.

Stein, P. E., Boodhoo, A., Armstrong, G. D., Cockle, S. A., Klein, M. H., & Read, R. J. (1994a) The crystal structure of pertussis toxin. *Structure.* **2**: 45-57.

Stein, P. E., Boodhoo, A., Armstrong, G. D., Heerze, L. D., Cockle, S. A., Klein, M. H., & Read, R. J. (1994b) Structure of a pertussis toxin-sugar complex as a model for receptor binding. *Nature. Structural Biology.* **1**: 591-596.

Steinman, L., Weiss, A., Adelman, N., Lim, M., Zuniga, R., Oehlert, J., Hewlett, E., & Falkow, S. (1985) Pertussis toxin is required for pertussis vaccine encephalopathy. *Proceedings of the National Academy Sciences of the USA.* **82**: 8733-8736.

Stenson, T. H. & Weiss, A. A. (2002) DsbA and DsbC are required for secretion of pertussis toxin by *Bordetella pertussis*. *Infection and Immunity.* **70**: 2297-2303.

Storsaeter, J., Olin, P., Renemar, B., Lagergard, T., Norberg, R., Romanus, V., & Tiru, M. (1988) Mortality and morbidity from invasive bacterial infections during a clinical trial of acellular pertussis vaccines in Sweden. *Pediatrics Infectious Diseases Journal.* **7**: 637-645.

Storsaeter, J., Hallander, H., Farrington, C. P., Olin, P., Mollby, R., & Miller, E. (1990) Secondary analyses of the efficacy of two acellular pertussis vaccines evaluated in a Swedish phase III trial. *Vaccine.* **8**: 457-461.

Storsaeter, J., Hallander, H. O., Gustafsson, L., & Olin, P. (1998) Levels of anti-pertussis antibodies related to protection after household exposure to *Bordetella pertussis*. *Vaccine.* **16**: 1907-1916.

Strnad, C. F. & Carchman, R. A. (1987) Human T lymphocyte mitogenesis in response to the B oligomer of pertussis toxin is associated with an early elevation in cytosolic calcium concentrations. *FEBS Letters.* **225**: 16-20.

Su, S. B., Silver, P. B., Wang, P., Chan, C. C., & Caspi, R. R. (2003) Dissociating the enhancing and inhibitory effects of pertussis toxin on autoimmune disease. *Journal of Immunology.* **171**, 2314-2319.

Taira, T., Saito, Y., Niki, T., Iguchi-Ariga, S. M., Takahashi, K., & Ariga, H. (2004) DJ-1 has a role in antioxidative stress to prevent cell death. *EMBO Reports.* **5**: 213-218.

Takasaki, S. & Kobata, A. (1986) Asparagine-linked sugar chains of fetuin: occurrence of tetrasialyl triantennary sugar chains containing the Gal beta 1----3GlcNAc sequence. *Biochemistry.* **25**: 5709-5715.

Tallett, A., Seabrook, R. N., Irons, L. I., Robinson, A., van Heyningen, S., & Atkinson, T. (1993) Localisation of a receptor-recognition domain on the S3 subunit of pertussis toxin by peptide mapping. *European Journal of Biochemistry.* **211**: 743-748.

Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M., & Ishii, S. (1982) Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. *Biochemistry.* **21**: 5516-5522.

Tamura, M., Nogimori, K., Yajima, M., Ase, K., & Ui, M. (1983) A role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of the biological effects on intact cells. *Journal of Biological Chemistry.* **258**: 6756-6761.

Tang, M. K., Wang, C. M., Shan, S. W., Chui, Y. L., Ching, A. K., Chow, P. H., Grotewold, L., Chan, J. Y., & Lee, K. K. (2006) Comparative proteomic analysis reveals a function of the novel death receptor-associated protein BRE in the regulation of prohibitin and p53 expression and proliferation. *Proteomics.* **6**: 2376-2385.

Thom, R. E. & Casnellie, J. E. (1989) Pertussis toxin activates protein kinase C and a tyrosine protein kinase in the human T cell line Jurkat. *FEBS Letters.* **244**: 181-184.

Thomas, M. G., Ashworth, L. A., Miller, E., & Lambert, H. P. (1989) Serum IgG, IgA, and IgM responses to pertussis toxin, filamentous hemagglutinin, and agglutinogens 2 and 3 after infection with Bordetella pertussis and immunization with whole-cell pertussis vaccine. *Journal of Infectious Diseases.* **160**: 838-845.

Thompson, W. E., Branch, A., Whittaker, J. A., Lyn, D., Zilberstein, M., Mayo, K. E., & Thomas, K. (2001) Characterization of prohibitin in a newly established rat ovarian granulosa cell line. *Endocrinology.* **142**: 4076-4085.

Tiru, M., Askelof, P., Granstrom, M., & Hallander, H. (1997) *Bordetella pertussis* serotype of clinical isolates in Sweden during 1970-1995 and influence of vaccine efficacy studies. *Developments in Biological Standardization.* **89** 239-245.

Tiwari, T., Murphy, T. V., & Moran, J. (2005) Recommended antimicrobial agents for the treatment and postexposure prophylaxis of pertussis: 2005 CDC Guidelines. *Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control.* **54**: 1-16.

Tome, D., Kozlowski, A., & Mabon. (1985) Carbon-13 NMR Study on the combination of Formaldehyde with Bovine Serum Albumin. *Journal of Agricultural and Food Chemistry.* **33**: 449-455.

Tome, D. & Naulet, N. (1981) Carbon 13 nuclear magnetic resonance studies on formaldehyde reactions with polyfunctional amino-acids. *International Journal of Peptide and Protein Research.* **17**: 501-507.

Townsend, R. R., Hilliker, E., Li, Y. T., Laine, R. A., Bell, W. R., & Lee, Y. C. (1982) Carbohydrate structure of human fibrinogen. Use of 300-MHz 1H-NMR to characterize glycosidase-treated glycopeptides. *Journal of Biological Chemistry.* **257**: 9704-9710.

Tuomanen, E. & Weiss, A. (1985) Characterization of two adhesins of *Bordetella pertussis* for human ciliated respiratory-epithelial cells. *Journal of Infectious Diseases.* **152**: 118-125.

Tuomanen, E., Towbin, H., Rosenfelder, G., Braun, D., Larson, G., Hansson, G. C., & Hill, R. (1988) Receptor analogs and monoclonal antibodies that inhibit adherence of *Bordetella pertussis* to human ciliated respiratory epithelial cells. *The Journal of Experimental Medicine.* **168**: 267-277.

Tyrrell, G. J., Peppler, M. S., Bonnah, R. A., Clark, C. G., Chong, P., & Armstrong, G. D. (1989) Lectin-like properties of pertussis toxin. *Infection and Immunity.* **57**: 1854-1857.

Tzima, E., Trotter, P. J., Orchard, M. A., & Walker, J. H. (1999) Annexin V binds to the actin-based cytoskeleton at the plasma membrane of activated platelets. *Experimental Cell Research.* **251**: 185-193.

Tzima, E., Trotter, P. J., Orchard, M. A., & Walker, J. H. (2000) Annexin V relocates to the platelet cytoskeleton upon activation and binds to a specific isoform of actin. *European Journal of Biochemistry.* **267**: 4720-4730.

Ui, M., Nogimori, K., Tamura, M. (1985) Islet-activating protein, pertussis toxin: subunit structure and mechanism for its multiple biological actions, pg 19-43 In: R.D. Sekura, J. Moss, M. Vaughan (Eds.), Academic press inc, London.

Ui, M. (1990) Pertussis Toxin as a Valuable Probe for G-protein Involvement in Signal Transduction, pg 45-77 In: Moss, J., Vaughan, M. (Eds). ADP-Ribosylating Toxins and G Proteins. Insights into Signal Transduction, American Society for Microbiology, Washington, D.C.

van't Wout, J., Burnette, W. N., Mar, V. L., Rozdzinski, E., Wright, S. D., & Tuomanen, E. I. (1992) Role of carbohydrate recognition domains of pertussis toxin in adherence of Bordetella pertussis to human macrophages. *Infection and Immunity.* **60**: 3303-3308.

van der Ark, A., van Straaten-van de Kappelle, Akkermans, A., Hendriksen, C., & van de, Donk, H. (1994) Development of pertussis serological potency test. Serological assessment of antibody response induced by whole cell vaccine as an alternative to mouse protection in an intracerebral challenge model. *Biologicals.* **22**: 233-242.

Van Dyk, D. D., Misztal, D. R., Wilkins, M. R., Mackintosh, J. A., Poljak, A., Varnai, J. C., Teber, E., Walsh, B. J., & Gray, P. P. (2003) Identification of cellular changes associated with increased production of human growth hormone in a recombinant Chinese hamster ovary cell line. *Proteomics.* **3**: 147-156.

van Meijeren, C. E., Vleeming, W., van de Kuil, T., Manni, J., Kegler, D., Hendriksen, C. F., & de Wildt, D. J. (2004a) In vivo pertussis toxin treatment reduces contraction of rat resistance arteries but not that of mouse trachea. *European Journal of Pharmacology.* **488**: 127-135.

van Meijeren, C. E., Vleeming, W., van de Kuil, T., Gerards, A. L., Hendriksen, C. F., & de Wildt, D. J. (2004b) Pertussis toxin-induced histamine sensitisation: an aspecific phenomenon independent from the nitric oxide system? *European Journal of Pharmacology.* **493**: 139-150.

van Meijeren, C. E., Vleeming, W., Dormans, J. A., van de Kuil, T., Opperhuizen, A., Hendriksen, C. F., & de Wildt, D. J. (2004c), Pertussis toxin relaxes small arteries with no vascular lesions or vascular smooth muscle cell injury. *Experimental and Toxicologic Pathology.* **56**: 139-143.

van Straaten-van de Kappelle, van der Gun, J. W., Marsman, F. R., Hendriksen, C. F., & van de Donk, H. J. (1997) Collaborative study on test systems to assess toxicity of whole cell pertussis vaccine. *Biologicals.* **25**: 41-57.

Veithen, A., Raze, D., & Locht, C. (2000) Intracellular trafficking and membrane translocation of pertussis toxin into host cells. *International Journal of Medical Microbiology.* **290**: 409-413.

Vidor, E., Hoffenbach, Agnes., & Plotkin, S. (1999) Pediatric Combination Vaccines, pg1-34 In: Ellis, R.W (Ed) Combination Vaccines: Development, Clinical Research, and Approval. Humana Press Inc, Totowa, New Jersey.

Vidor, E., Hoffenbach, A., & Fletcher, M. A. (2001) *Haemophilus influenzae* type b vaccine: reconstitution of lyophilised PRP-T vaccine with a pertussis-containing paediatric combination vaccine, or a change in the primary series immunisation schedule, may modify the serum anti-PRP antibody responses. *Current Medical Research and Opinion.* **17**: 197-209.

Vincent, J. P., Lazdunski, M., & Delaage, M. (1970) On the use of tetranitromethane as a nitration reagent. The reaction of phenol side-chains in bovine and porcine trypsinogens and trypsins. *European Journal of Biochemistry.* **12**: 250-257.

Vipond, C., Suker, J., Jones, C., Tang, C., Feavers, I. M., & Wheeler, J. X. (2006) Proteomic analysis of a meningococcal outer membrane vesicle vaccine prepared from the group B strain NZ98/254. *Proteomics.* **6**: 3400-3413.

von Kries, R., Toschke, A. M., Strassburger, K., Kundi, M., Kalies, H., Nennstiel, U., Jorch, G., Rosenbauer, J., & Giani, G. (2005) Sudden and unexpected deaths after the administration of hexavalent vaccines (diphtheria, tetanus, pertussis, poliomyelitis, hepatitis B, *haemophilius influenzae* type b): is there a signal? *European Journal of Pediatrics.* **164**: 61-69.

Wardlaw, A. C. & Parton, R. (1982) *Bordetella pertussis* toxins. *Pharmacology and Therapeutics.* **19**: 1-53.

Wardlaw, A. C., & Parton R. (1983) *Bordetella pertussis* toxins, pg. 327–380. In: F. Dorner and J. Drews (ed.), Pharmacology of bacterial toxins. Pergamon Press Ltd., Oxford, England.

Watanabe, M., Takimoto, H., Kumazawa, Y., & Amano, K. (1990) Biological properties of lipopolysaccharides from Bordetella species. *Journal of General Microbiology.* **136**: 489-493.

Watanabe, M., Komatsu, E., Sato, T., & Nagai, M. (2002) Evaluation of efficacy in terms of antibody levels and cell-mediated immunity of acellular pertussis vaccines in a murine model of respiratory infection. *FEMS Immunology and Medical Microbiology.* **33**: 219-225.

Weiss, A.A. (1997) Mucosal immune defences and the response of *Bordetella pertussis*. *American Society for Microbiology News.* **63**: 22-27.

Weiss, A. A. & Falkow, S. (1984) Genetic analysis of phase change in *Bordetella pertussis*. *Infection and Immunity.* **43**: 263-269.

Weiss, A. A. & Goodwin, M. S. (1989) Lethal infection by *Bordetella pertussis* mutants in the infant mouse model. *Infection and Immunity.* **57**: 3757-3764.

Weiss, A. A., Johnson, F. D., & Burns, D. L. (1993) Molecular characterization of an operon required for pertussis toxin secretion. *Proceedings of the National Academy Sciences of the USA.* **90**: 2970-2974.

Wen, Y., Eng, C. H., Schmoranzer, J., Cabrera-Poch, N., Morris, E. J., Chen, M., Wallar, B. J., Alberts, A. S., & Gundersen, G. G. (2004) EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. *Nature Cell Biology.* **6**: 820-830.

Westermeier, R. & Marouga, R. (2005) Protein detection methods in proteomics research. *Bioscience Reports.* **25**: 19-32.

WHO (1998), Guidelines for the production and control of the acellular component of monovalent or combined vaccines. In: WHO Technical Report Series. **878**, 57-72.

WHO position paper (2005) *Weekly Epidemiological Record.* **80**: 29–40. (http://www.who.int/wer)

WHO Model Prescribing Information (2001) Drugs used in bacterial infections. World Health Organization (ordering code: WHO/V&B/02.28).

WHO (2005) Recommendations for whole cell vaccines. Annex 6, 56th meeting of the ECBS. (http://www.who.int/biologicals/publications/ECBS%202005%20Annex%206%20Pertusss.pdf)

Wildgruber, R., Harder, A., Obermaier, C., Boguth, G., Weiss, W., Fey, S. J., Larsen, P. M., & Gorg, A. (2000) Towards higher resolution: two-dimensional electrophoresis of Saccharomyces cerevisiae proteins using overlapping narrow immobilized pH gradients. *Electrophoresis.* **21**: 2610-2616.

Willems, R., Paul, A., van der Heide, H. G., ter Avest, A. R., & Mooi, F. R. (1990) Fimbrial phase variation in Bordetella pertussis: a novel mechanism for transcriptional regulation. *EMBO Journal.* **9**: 2803-2809.

Willems, R. J., Geuijen, C., van der Heide, H. G., Matheson, M., Robinson, A., Versluis, L. F., Ebberink, R., Theelen, J., & Mooi, F. R. (1993) Isolation of a putative fimbrial adhesin from *Bordetella pertussis* and the identification of its gene. *Molecular Microbiology.* **9**: 623-634.

Willems, R. J., Geuijen, C., van der Heide, H. G., Renauld, G., Bertin, P., van den Akker, W. M., Locht, C., & Mooi, F. R. (1994) Mutational analysis of the *Bordetella pertussis* fim/fha gene cluster: identification of a gene with sequence similarities to haemolysin accessory genes involved in export of FHA *Molecular Microbiology.* **11**: 337-347.

Williams, J. & Lowe, J. M. (1971) The cross-linking of tyrosine by treatment with tetranitromethane. *Biochemical Journal.* **121**: 203-209.

Wilson, R., Read, R., Thomas, M., Rutman, A., Harrison, K., Lund, V., Cookson, B., Goldman, W., Lambert, H., & Cole, P. (1991) Effects of *Bordetella pertussis* infection on human respiratory epithelium in vivo and in vitro. *Infection and Immunity.* **59**: 337-345.

Witvliet, M. H., Burns, D. L., Brennan, M. J., Poolman, J. T., & Manclark, C. R. (1989) Binding of pertussis toxin to eucaryotic cells and glycoproteins. *Infection and Immunity.* **57**: 3324-3330.

Witvliet, M. H., Vogel, M. L., Wiertz, E. J., & Poolman, J. T. (1992) Interaction of pertussis toxin with human T lymphocytes. *Infection and Immunity.* **60**: 5085-5090.

Wolff, J., Cook, G. H., Goldhammer, A. R., & Berkowitz, S. A. (1980) Calmodulin activates prokaryotic adenylate cyclase. *Proceedings of the National Academy sciences of the USA.* **77**: 3841-3844.

Wong, C. M., Chun, A. C., Kok, K. H., Zhou, Y., Fung, P. C., Kung, H. F., Jeang, K. T., & Jin, D. Y. (2000) Characterization of human and mouse peroxiredoxin IV: evidence for inhibition by Prx-IV of epidermal growth factor- and p53-induced reactive oxygen species. Antioxidants & Redox Signaling. **2**: 507-518.

Wong, W. S. & Rosoff, P. M. (1996) Pharmacology of pertussis toxin B-oligomer. *Canadian journal of Physiology and Pharmacology.* **74**: 559-564.

Wortis, N., Strebel, P. M., Wharton, M., Bardenheier, B., & Hardy, I. R. (1996) Pertussis deaths: report of 23 cases in the United States, 1992 and 1993. *Pediatrics*, **97**: 607-612.

Xing, D., Das, R. G., O'Neill, T., Corbel, M., Dellepiane, N., & Milstien, J. (2001) Laboratory testing of whole cell pertussis vaccine: a WHO proficiency study using the Kendrick test. *Vaccine.* **20**: 342-351.

Xing, D., Canthaboo, C., Douglas-Bardsley, A., Yuen, C. T., Prior, S., Liu, Y., & Corbel, M. (2002) Developments in reduction and replacement of in vivo toxicity and potency tests for pertussis vaccines *Developmental Biology. (Basel).* **111**: 57-68.

Xing, D. K., Canthaboo, C., & Corbel, M. J. (1998) Nitric oxide induction in murine macrophages and spleen cells by whole-cell *Bordetella pertussis* vaccine. *Vaccine.* vol. **16**: 16-23.

Xing, D. K., Das, R. G., Williams, L., Canthaboo, C., Tremmil, J., & Corbel, M. J. (1999) An aerosol challenge model of *Bordetella pertussis* infection as a potential bioassay for acellular pertussis vaccines. *Vaccine.* **17**: 565-576.

Xing, D., Das, R. G., Newland, P., & Corbel, M. (2002) Comparison of the bioactivity of reference preparations for assaying *Bordetella pertussis* toxin activity in vaccines by the histamine sensitisation and Chinese hamster ovary-tests: assessment of validity of expression of activity in terms of protein concentration. *Vaccine.* **20**: 3535-3542.

Xu, Y. & Barbieri, J. T. (1995) Pertussis toxin-mediated ADP-ribosylation of target proteins in Chinese hamster ovary cells involves a vesicle trafficking mechanism. *Infection and Immunity.* **63**: 825-832.

Xu, Y. & Barbieri, J. T. (1996) Pertussis toxin-catalyzed ADP-ribosylation of Gi-2 and Gi-3 in CHO cells is modulated by inhibitors of intracellular trafficking. *Infection and Immunity.* **64**: 593-599.

Yaari, E., Yafe-Zimerman, Y., Schwartz, S. B., Slater, P. E., Shvartzman, P., Andoren, N., Branski, D., & Kerem, E. (1999) Clinical manifestations of *Bordetella pertussis* infection in immunized children and young adults. *Chest.* **115**: 1254-1258.

Yajima, M., Hosoda, K., Kanbayashi, Y., Nakamura, T., Takahashi, I., & Ui, M. (1978) Biological properties of islets-activating protein (IAP) purified from the culture medium of *Bordetella pertussis*. *Journal of Biochemistry.(Tokyo).* **83**: 305-312.

Yamamoto, A., Nagata, N., Ochiai, M., Kataoka, M., Toyoizumi, H., Okada, K., & Horiuchi, Y. (2002) Enhanced sensitisation of mice with diphtheria tetanus acellular pertussis vaccine to local swelling reaction to the booster immunisation. *Vaccine.* **20**: 3088-3094.

Yan, J. X., Wait, R., Berkelman, T., Harry, R. A., Westbrook, J. A., Wheeler, C. H., & Dunn, M. J. (2000) A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. *Electrophoresis.* **21**: 3666-3672.

Yeh, S. H. (2005) Pediarix: clinical trials. *Expert Review of Vaccines.* **4**: 139-145.

Yokota, S., Yanagi, H., Yura, T., & Kubota, H. (2001) Cytosolic chaperonin-containing t-complex polypeptide 1 changes the content of a particular subunit species concomitant with substrate binding and folding activities during the cell cycle. *European Journal of Biochemistry.* **268**: 4664-4673.

Yong, T., Meininger, G. A., & Linthicum, D. S. (1993) Enhancement of histamine-induced vascular leakage by pertussis toxin in SJL/J mice but not BALB/c mice. *Journal of Neuroimmunology.* **45**: 47-52.

Yoshima, H., Matsumoto, A., Mizuochi, T., Kawasaki, T., & Kobata, A. (1981) Comparative study of the carbohydrate moieties of rat and human plasma alpha 1-acid glycoproteins. *Journal of Biological Chemistry.* **256**: 8476-8484.

Yuen, C. T., Canthaboo, C., Menzies, J. A., Cyr, T., Whitehouse, L. W., Jones, C., Corbel, M. J., & Xing, D. (2002) Detection of residual pertussis toxin in vaccines using a modified ribosylation assay. *Vaccine.* **21**: 44-52.

Zhang, J. M., Cowell, J. L., Steven, A. C., Carter, P. H., McGrath, P. P., & Manclark, C. R.(1985) Purification and characterization of fimbriae isolated from *Bordetella pertussis Infection and Immunity.* **48**: 422-427.

Zinka, B., Rauch, E., Buettner, A., Rueff, F., & Penning, R. (2006) Unexplained cases of sudden infant death shortly after hexavalent vaccination. *Vaccine*. **24**: 5779-5780.