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Identification of gene markers for *in vitro* toxicity control testing of pertussis vaccines

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A mon grand-père, Ildevert Moncassin (1924-2004),

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%	Percentage
Ab	antibody
ACT	adenylate cyclase toxin
ACV	acellular pertussis vaccine
ADP	adenosine diphosphate
Ag	antigen
APCs	antigen-presenting cells
API	apoptosis protein inhibitor
Arg	arginine
Asp	asparagine
ATP	adenosine triphosphate
BSA	bovine serum albumin
Bvg	*Bordetella* virulence gene
°C	degrees Celsius
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CHRNA	cholinergic receptor, nicotinic, alpha polypeptide 2
CHO	chinese hamster ovary cell
Ci	curie (µCi=microCurie)
CMI	cell-mediated immunity
CR3	complement receptor 3
CRE-Bp1	cAMP response element-binding protein mRNA
CTL	cytotoxic T lymphocyte
dATP	2'-deoxyadenosine 5'-triphosphate
DC	dendritic cell
dCTP	2'-deoxycytosine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMEM/F12	Dulbecco's minimum essential media supplemented with Ham's F12
DNA	deoxyribonucleic acid
DNT	dermonecrotic toxin
DPH2L1	diphthamide biosynthesis protein 2
dPT	detoxified pertussis toxoid
DTH	delayed type hypersensitivity
DTaP	diphtheria-tetanus-acellular pertussis vaccine
dTTP	2'-deoxytyrosine 5'-triphosphate
DTT	dithiothreltol
DTwP	diphtheria-tetanus-whole cell pertussis vaccine
EBM	endothelial cells basal media
ED_{50}	effective dose 50%
EF	elongation factor
ELISA	enzyme-linked immunosorbent assay
EP	European pharmacopoeia
ER	endoplasmic reticulum
E-selectin	endothelial adhesion molecule 1
FCS	fetal calf serum
Fim2	serotype-2 fimbriae
Fim3	serotype-3 fimbriae
FITC	fluorescein isothiocyanate
FHA	filamentous haemagglutinin

G	gram
Galectin 3	lectin, galactoside-binding, soluble, 3 binding protein
GapDH	glyceraldehyde-3-phosphate dehydrogenase
$G_{i\alpha}$ subunit	inhibitory alpha-subunit of guanyl nucleotide binding protein
gly	glycine
G-protein	guanyl nucleotide binding protein
GRO	macrophage inflammatory protein
H	hour(s)
HEPES	1-Piperazineethane sulfonic acid, 4-(2-hydroxyethyl)- monosodium salt
HIST	histamine sensitisation test
HUVEC	human umbilical vein endothelial cell
IC	intracerebral challenge
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
i.m.	Intra-muscular
IU	International unit
KDa	kilodalton
LAL	*Limulus* amoebocyte lysate
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LRI	leucocyte response integrin
Lys	lysine
M	molar
MCP	macrophage chemoattractant protein
MEM	minimum essential medium
µg	microgram
mg	milligram
MHC	major histocompatibility complex
min	minutes
MIP	macrophage inflammatory protein
ml	millilitres
µm	micrometre
mM	millimolar
MΦ	macrophage
mRNA	messenger ribonucleic acid
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
Mw	molecular weight
MWGT	mouse weight-gain test
NAD	nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NF-κβ	nuclear factor kappa beta
NIBSC	National Institute for Biological Standards and Control
NK	natural killer
Nm	nanometre
NO	nitric oxide
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pg	picogram

pMol	picomolar
PRN	pertactin
PT	pertussis toxin
ptl	pertussis toxin liberation genes
RGD	arginine-glycine-asparagine
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
RTX	repeats in toxin
SAR1	GTP-binding protein Sara
SCY	Small cytokine
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Taq	*Thermophilus aquaticus*
TBE	Tris-borate EDTA buffer
TCR	T cell receptor
TCT	tracheal cytotoxin
TCF	tracheal colonization factor
TGF	tumour growth factor
Th-1 or-2	T helper cell type 1 or 2
Thp	T helper cell precursor
TNF	tumour necrosis factor
TNFAIP	tumour necrosis factor alpha induce protein
UK	United Kingdom
Vag	virulence-activated gene
VCAM-1	vascular cell adhesion molecule-1
VIP	vasoactive intestinal peptide
VLA-5	integrin alpha 5
Vrg	virulence-repressed gene
v/v	volume to volume
WHO	World Health Organisation
WCV	whole-cell vaccine
w/v	weight to volume

Abstract

Whole-cell (WCV) and acellular (ACV) pertussis vaccines play an essential role in immunization programs for the prevention of the disease pertussis (whooping cough). Pertussis toxin (PT), one of the *Bordetella pertussis* toxins, is a major component in both WCV and ACV. The histamine sensitisation test (HIST) is a toxicity test, in mice, used to assure the absence of significant residual PT activity in pertussis vaccines. HIST is a lethal test and large variations in test performance have been observed. The objective of this project was to investigate the gene expression of selected cell lines treated with PT and detoxified pertussis toxin (dPT) using microarray technology and, from the expression profiles obtained, to identify gene markers of the toxicological effects of PT which may form the base to develop an *in vitro* assay as an alternative to HIST. Two cell types were investigated: human umbilical vein endothelial cells (HUVEC) and bronchial epithelial cells (NL20). Based on morphological and cytotoxicity studies, the cells were treated with PT and dPT at a concentration of 2.5 µg/ml for 6h. The gene expression profiles obtained lead to the speculation that PT could play an important role in the induction of the cell-mediated immunity (up-regulation of: galectin 3, small inducible cytokine subfamily 20, Thy-1 cell surface antigen and CD63 genes) and that PT could also have an important role in the induction of vascular permeability (up-regulation of: platelet-derived growth factor, vascular endothelial growth factor c) and that the effect may take place at the brain level (up-regulation of: glial fibrillary acidic protein, chlorine channel 3, cholinergic receptor). RT-PCR study needs further investigation, nevertheless, the ICAM 1 gene (specifically up-regulated by PT + TNF-α treatment) may serve as a gene marker of PT toxicity.In addition, data from a cell migration study with endothelial cells suggested that PT could be involved in angiogenesis. The phenotypic and genomic data presented in this study suggest that, in order to develop a replacement for the *in vivo* HIST control test, an *in vitro* permeability assay with monolayer endothelial cells may be worth investigating.

1. INTRODUCTION

1.1. Pathogenesis of whooping cough

Bordetella pertussis is a Gram-negative bacterium and the causative agent of whooping cough or pertussis. The disease mainly affects young children displaying a protracted course that is measured in weeks. It is characterised by the development of vigorous paroxysmal coughing, often associated with vomiting and, occasionally, with brain damage. At the beginning of the 20th century, pertussis was a major cause of infant mortality worldwide. Largely due to immunization, it is presently of less consequence in developed countries but continues to be a major child health problem in developing nations (Mortimer, 1998).

The introduction of *B. pertussis* into the respiratory tract is followed by interaction with ciliated epithelial cells, likely to be dependent on the bacterial adhesins filamentous haemagglutinin (FHA), fimbriae, a 69-kDa protein named pertactin (PRN), tracheal colonization factor (TCF) and the serum resistance protein BrkA. Expression of these virulence factors is regulated in response to environmental stimuli by a two-component regulatory system encoded by the *bvg* genes (Uhl & Miller, 1995). The next step in pathogenesis is thought to be paralysis of the cilia and death of ciliated cells, probably mediated by a synergistic effect of tracheal cytotoxin (TCT) and lipopolysaccharide (LPS) via induction of interleukin-1 (IL-1) and nitric oxide (Flak & Goldman, 1996; 1999). This leads to the inactivation of the mucociliary clearance mechanism and may allow the bacteria to remain in the respiratory tract although it presumably triggers the severe coughing that is characteristic of pertussis. In addition, the expression of other virulence factors such as pertussis toxin (PT) (Carbonetti *et al.*, 2003) and adenylate cyclase-toxin (ACT) causes further damage

and also interference with immune responses. Depending on the response of the patient, the infection will be cleared over time or may progress, in some cases, to pertussis pneumonia and possibly death (Preston & Maskell, 2002).

1.2. Epidemiology and burden of Pertussis disease

The introduction of pertussis vaccines and their subsequent widespread use globally have resulted in a reduction in the incidence, morbidity and mortality of this disease in many countries (de Melker *et al.*, 1997). However, globally, 20-40 million cases of pertussis still occur each year, 90% of which are in developing countries, and there are up to 400,000 fatalities each year, mostly in young infants (Forsyth *et al.*, 2004, www.WHO.int/vaccines-diseases/pertussisvaccine.shtml). Furthermore, during the past several decades, despite high childhood vaccination coverage levels for pertussis vaccine, *B.pertussis* infection has been increasingly recognized worldwide as a significant cause of cough illness in adolescents and adults (MMWR, 2005). Overall an increased incidence of infant, adolescent and adult pertussis has been observed worldwide since the introduction of widespread vaccination. This is of concern because adolescents and adults have been identified as a source of transmission of pertussis to very young infants who are unimmunized or partially immunized and thus more vulnerable to disease-related complications and higher mortality. A number of hypotheses have been put forward to explain the observed disease resurgence, including waning natural and vaccine-induced immunity and a lack of natural boosting, a reduction in vaccine coverage or poor vaccine immunogenicity and efficacy and the emergence of new bacterial variants (Caro et *al.*, 2005).

1.3. *Bordetella pertussis* virulence factors

Understanding the disease, its epidemiology and in developing an optimum vaccine has been difficult. Many virulence factors have been purified and characterised at a molecular level. They can be grouped into two different categories: the adhesins and toxins. The major adhesins include filamentous haemagglutinin (FHA), fimbriae, a 69-kDa protein named pertactin (PRN), tracheal colonization factor (TCF) and the serum resistance protein BrkA. In addition to the adhesins, *Bordetella pertussis* produces a number of toxins, i.e. pertussis toxin (PT), adenylate cyclase toxin (ACT), dermonecrotic toxin (DNT), tracheal cytotoxin (TCT) and endotoxin lipopolysaccharide (LPS). The cellular location of those, and some other, virulence factors and their mechanisms of secretion are shown in Figure 1.

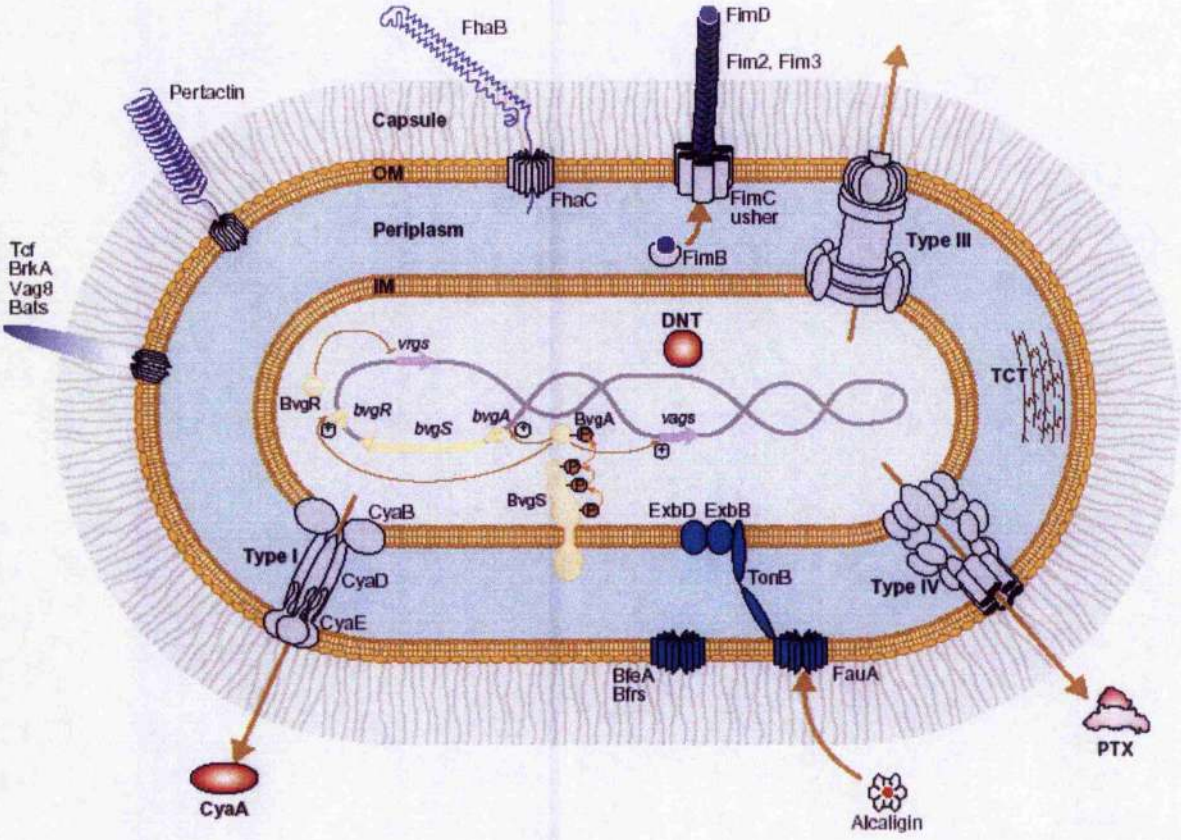


Figure 1. *Bordetella pertussis* virulence factors

Bordetella pertussis is depicted as a Gram-negative organism with inner and outer membranes (IM and OM), a periplasm and a capsule. The adhesins Fim, FhaB, pertactin, Tcf, BrkA are shown in blue; the toxins PT, ACT, CyaA and DNT are in red, the accessory proteins FhaC, FimB, FimC, Type III, Type IV and Type I are in grey; and the regulatory systems BvgA, BvgS and BvgR are in beige. The large brown arrows represent the orientation of export and import of virulence factors and siderophores, respectively. The thinner brown arrows show the phosphorelay and the regulation circuit (Locht *et al.*, 2001).

1.3.1. Pertussis toxin (PT)

1.3.1.1. Structure

Pertussis toxin (PT) is representative of the A-B subunit class of bacterial exotoxins. Another member of this group includes cholera toxin (Tamura *et al.*, 1982). The toxin comprises an enzymically active A subunit (S1) and a B oligomer, made up of 5 subunits (S2-S4 and S3-S4 dimers connected by S5)(Tamura *et al.*, 1982; 1983).

1.3.1.2. Gene transcription, protein synthesis and excretion.

The genes encoding the PT subunits S1-S5 are called *ptxA-E* and a number of polymorphisms in the genes encoding the S1 and S3 subunits (*ptxA* and *ptxC)* have been revealed (van Amersfoorth *et al.*, 2005). The secretion of PT is dependent on the expression of several genes called *ptl* (for pertussis toxin liberation). It has been reported that the *ptl* genes probably constitute, with the *ptx* genes, a single polycistronic operon, composed of the five *ptx* genes followed by nine *ptl* genes (Baker *et al.*, 1995; Kotob *et al.*, 1995; Farizo *et al.*, 1996). The biogenesis of PT includes synthesis of the individual subunits and their transport through the inner membrane probably via the Sec machinery. After cleavage of the signal peptides, the toxin is assembled in the periplasm and fully assembled holotoxin is secreted through the outer membrane (Farizo *et al.*, 2000) via a complex secretion apparatus, often referred to as a type IV secretion system (Burns, 1999)(See Figure 1).

1.3.1.3. Mechanism of action

Several experimental data suggest that PT entry into target cells follows the retrograde transport system. Subcellular fractionation experiments showed that PT travels to the Golgi complex following receptor-mediated endocytosis (el Baya *et al.*, 1997). PT may possibly travel all the way to the ER, where it may be activated by the

dissociation of S1 from the B oligomer, and then the enzymatically active subunit translocates into the cytosol.

1.3.1.4. Pathophysiological effects of PT

The majority of the biological effects of PT are due to the activity of the enzyme subunit S1 that transfers an adenosine diphosphate ribose (ADP-ribose) moiety from NAD^+ to the α-subunits of the Gi/o proteins of the family of signal-transducing guanine-nucleotide-binding proteins (G-proteins) which are involved in eukaryotic signal transduction (Gierschik, 1992). This ADP-ribosylation generally leads to an uncoupling of the modified G-protein from the corresponding receptor and the loss of effector regulation, ultimately leading to adverse effects on the target cell and adverse reactions in the host.

The holotoxin produces a wide range of pathophysiological effects including insulin secretion by activation of the islets of Langerhans, β-adrenoreceptor blockadge, histamine sensitisation, lymphocytosis, mitogenesis, melanocyte stimulation, T and B-lymphocytes stimulation, inhibition of neutrophil chemotaxis and monocyte migration (Tamura *et al.*, 1982; Sekura, 1985; Munoz, 1985; 1998). On dendritic cells, PT and genetically-detoxified PT have been reported to induce interleukin 12 production (Ausiello *et al.*, 2002).

1.3.1.5. Cell-binding properties of PT and its consequences

Once PT reaches the extrabacterial environment, it can interact with specific receptors on host target cells. This binding occurs via the B oligomer. Both dimers (S2-S4, S3-S4) of the B oligomer have been associated with the binding process and there is evidence of different binding specificities (Nogimori *et al.*, 1986; Witvliet *et al.*, 1989).

Studies carried out on the PT holotoxin (A and B oligomers)

Virtually all mammalian cells that have been studied so far contain PT receptors on their surface. However, a distinct universal receptor has not been identified. Rather, different cell types may express different PT receptors. Nevertheless, a common feature of PT receptors is that they are glycoconjugates, generally sialoglycoproteins (Armstrong *et al.*, 1988). In addition, PT can bind to a 43-kDa plasma-membrane protein of T lymphocytes and can also bind to CD14 (Zhang *et al.*, 1995; Li & Wong, 2000). Chinese hamster ovary (CHO) cells contain a 165-KDa glycoprotein at their surface recognized by PT or purified B oligomer in a lectin-like manner. Specificity for sialidated N-linked oligosaccharide of the protein may be involved in the binding to the cell surface receptor (Brennan *et al.*, 1988). The binding properties of PT have been studied and used to develop a cellular-based method to investigate its toxicity. By binding on the CHO cell surface, PT is responsible for the resulting cell clustering in a dose-dependent manner (Brennan *et al.*, 1988). PT binds to a 70-kDa protein on human lymphocytes (Clark & Armstrong, 1990). This protein may be related to the 73-kDa LPS receptor. However, LPS and PT bind different domains of the 73-kDa protein (Lei & Morrison, 1993) on the cell surface of murine splenocytes. The toxin was also found to induce expression of IL-2-receptor on CD3+ cells and to stimulate IL-2 production. PT induced proliferation of both CD4+ and CD8+ T cells and stimulates IL-1 production by monocytes in the presence (but not in the absence) of accessory cells (Grenier-Brossette *et al.*, 1991).

Studies carried out on the B oligomer

Studies with the human T-lymphocyte Jurkat cell line have shown that toxoids or the purified B subunit of PT bind specifically to a 43-kDa membrane protein (p43) and induce synthesis of second messenger. Both toxoids and B subunit are devoid of enzyme activity but are as potent as the holotoxin in this action. The p43 may be a

novel T-cell membrane protein coupled to the CD3-TCR complex signal transduction apparatus (Rogers *et al.*, 1990). Among the early events, that occur as a consequence of TCR occupancy are increased intracellular [Ca^{2+}] (Tsien *et al.*, 1982; Weiss *et al.*, 1984), K^+ efflux (Russell & Dobos, 1983), Cl^- influx (Russell & Dobos, 1983) and the simultaneous production of inositol polyphosphates and diacylglycerol (Imboden & Stobo, 1985). B oligomer binding was found to induce expression of IL-2 receptor on $CD3^+$ cells and to stimulate IL-2 production. PT induced proliferation of both $CD4^+$ and $CD8^+$ T cells in the presence of accessory cells. In addition B oligomer alone can trigger phospholipase C and tyrosine kinase-dependent signal transduction, unrelated to the mechanisms of G_i ribosylation events, suggesting an important role in cell activation (Wong & Rosoff, 1996).

These events may, in some manner, help to provide the signal for functional outcomes of PT actions such as cell proliferation or cell-mediated cytolysis.

1.3.2. Detoxified PT

PT is rendered non-toxic either by chemical or genetic detoxification (di Tommaso *et al.*, 1994). The general approach for inactivation of the bacterial antigens present in pertussis vaccines has consisted of different chemical treatments, such as with formaldehyde, hydrogen peroxide, or teranitromethane (Edwards *et al.*, 1995). Formaldehyde treatment is a process widely used in vaccine preparation to inactivate toxin molecules such as PT and to stabilize protein components (Sato *et al.*, 1984). Formaldehyde reacts mainly with ε-amino groups of lysine residues to give an unstable product that can then react with a second amino group to form a stable methylene bridge. These reactions can occur either between amino acids of the same molecule, resulting in internal cross-linking of the protein, or between two molecules, resulting in dimerization (di Tommaso *et al.*, 1994). Generally, vaccines

treated with formaldehyde have proven very effective in inducing protective antibody responses, but it has also been reported that formaldehyde treatment could cause reductions in antigen processing, binding affinities of raised antibodies and potency (di Tommaso *et al.*, 1994; Bolgiano *et al.*, 1999). Also, the reversion to toxin of detoxified PT has been observed after mild treatment of PT with formalin and further incubation of the formalin-detoxified PT at 37^0C for three weeks (Kataoka *et al.*, 2002). As explained below, chemically-detoxified PT has limitations that have been counteracted by the development of genetically detoxified PT.

The feasibility of a genetic approach in the development of new vaccines has been demonstrated by the production of genetically-detoxified PT, which has been shown to be efficacious in preventing pertussis in infants. Genetic detoxification appears to leave intact most of the pathophysiological and immunological effects mediated by the B oligomer (Wong & Rosoff, 1996). The mutant *B. pertussis* strains created by homologous recombination, produce PT that contains amino acid substitutions in the S1 subunit. These mutant forms of the toxin are known as 9K/129G, 13L/129G, and 26I/129G, and all contain the Glu-129→Gly substitution in addition to Arg9→Lys, Arg13→Leu, or Trp-26→Ile substitutions, respectively (Pizza *et al.*, 1989). These toxin mutants are devoid of any enzymatic activity. PT 9K/129G did not induce leukocytosis, nor insulin secretion, nor did it cause death by sensitisation to histamine (Nencioni *et al.*, 1990). Other studies have demonstrated that the genetically-inactivated PT was also devoid of other toxic properties of the wild type PT. For example, as compared to wild type PT, the mutant had greatly reduced ability to induce IgE *in vitro* (Van der Pouw-Kraan *et al.*, 1995) and *in vivo* (Kosecka *et al.*, 1994; Roberts *et al.*, 1995), to induce long-lasting enhancement of nerve-mediated intestinal permeabilization and antigen uptake (Kosecka *et al.*, 1994), to

inhibit IL-1 mediated IL-2 release in EL4 6.1 thymoma cells (Zumbihl *et al.*, 1995), and to inhibit neutrophil migration (Brito *et al.*, 1997). However, the non-toxic properties of PT, such as T-cell mitogenicity, haemagglutination (Nencioni *et al.*, 1990), platelet activation (Sindt *et al.*, 1994) and mucosal adjuvanticity (Roberts *et al.*, 1995) were still conserved in the mutant PT.

1.3.3. Adenylate cyclase toxin (ACT)

Adenylate cyclase toxin is a bifunctional protein of 1706 amino acids. The protein carries both calmodulin-dependent adenylate cyclase enzymatic and pore-forming (e.g. haemolytic) activities (Ladant & Ullmann, 1999). The cyclase domain is located at the N terminus of the protein whereas the pore-forming domain is located at the C terminus. Once inside the host cell, the protein can express its calmodulin-dependent adenylate cyclase activity. By catalysing the conversion of cytoplasmic ATP into cAMP, the intracellular level of cAMP increases, which disturbs the normal function of the cell (Confer & Eaton, 1982). ACT has been reported to inhibit chemotaxis, phagocytosis, superoxide generation in polymorphonuclear leukocytes and to induce apoptosis in J774A macrophages (Friedman *et al.*, 1987; Khelef & Guiso, 1995). In addition, ACT has been shown to trigger apoptosis in murine macrophages both *in vitro* and *in vivo* (Gueirard *et al.*, 1998). As ACT can modify host defences, it is thought that it might play a role in the early stages of the infection.

Adenylate cyclase toxin is a member of the RTX (repeats in toxins) toxin family. However, its specific haemolytic activity is rather low compared with that of the other members of the family (Bellalou *et al.*, 1990), suggesting that the main role of the haemolytic domain is not to lyse red blood cells, but to deliver the catalytic domain into target cells. Also, ACT is synthesised in an inactive form and requires a cytosolic palmytoylation of the Lys983 residue by the CyaC protein to express the complete

cell invasive activity. The palmytoylation is required for the binding and the internalisation of the ACT into the target cell (Gray *et al.*, 1999). Once active, the toxin is excreted via a type I system involving accessory proteins called CyaB, CyaD and CyaE (Glaser *et al.*, 1988)(Figure 1).

1.3.4. Dermonecrotic toxin (DNT)

The dermonecrotic toxin is also called heat-labile toxin as it is inactivated by incubation at 56°C for 60 minutes (Livey & Wardlaw, 1984). This toxin is not secreted by the bacteria and its location is intracellular (Cowell *et al.*, 1979). The toxin has an A-B structure with the receptor-binding B subunit at the N terminal and the enzymatic active site at the C terminal end. The protein is dermonecrotic, induces splenic atrophy and, when injected intravenously in its purified form to a mouse, it is lethal. However, its role in the biology of the disease is not clearly understood. At a molecular level, DNT causes both deamination and polyamination of Rho protein in target cells, affecting its GTPase activity and thereby constitutively activating the protein (Masuda *et al.*, 2000). As it is very difficult to raise antibodies against the toxin, it has not been used as a vaccine component.

1.3.5. Lipopolysaccharide (LPS)

Bordetella pertussis LPS resolves as two bands designated A and B, when separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Band B is composed of lipid A and a branched-chain core oligosaccharide. Addition of a trisaccharide to the band B form creates a larger molecule (LOS, lipooligosaccharide), referred to as band A (Harvill *et al.*, 2000). Unlike the highly polymerized LPS in some other bacteria, *B. pertussis* LPS (or LOS) does not appear to protect the bacterium from complement killing. Is has been reported that *B.*

pertussis endotoxin may be involved in the whooping cough syndrome by inducing NO production and release by tracheal cells, thereby poisoning the activity of adjacent ciliated cells (Flak & Goldman, 1999). Monoclonal antibodies to band A LPS have been shown to be bactericidal (Shahin *et al.*, 1994). However, LPS has been intentionally removed from the new acellular vaccines because of the potential toxicity of the lipid A component. If the toxic lipid A portion of the LPS could be separated from the protective portion, this antigen may be safe for vaccine use.

A synergistic effect between LPS and PT has been reported. Indeed, by binding the 73-kDa protein receptor, LPS and PT increase the production of interleukin 12 by dendritic cells. Dendritic cells may represent the link between the innate and the adaptive immune response, as shown by secretion of chemotactic proteins and regulatory cytokines that attract natural immune effectors and drive specific immune responses. Synergy between PT and LPS, in enhancing IL-12 production, might be relevant for the mechanisms of vaccine-induced protection of whole-cell vaccines (Ausiello *et al.*, 2002).

A possible synergistic activity between LPS and FHA has also been noted in the FHA-associated TNF-α secretion and apoptosis in monocytes. However, unlike *E.coli* LPS which has been associated with release of TNF-α by monocytes, such evidence has not yet been found for LPS from *B. pertussis* (Njamkepo *et al.*, 2000).

As pertussis is not usually a febrile disease, any action of LPS in the natural infection may to be localized to the respiratory tract. Nevertheless, its pyrogenicity is relevant in the context of vaccines. The reactogenicity of the whole-cell vaccine has been attributed to the content of active PT and LPS and hence these require careful monitoring (Redhead & Seagroatt, 1986; Gupta *et al.*, 1988). The World Health Organization issued its guidelines for acellular pertussis vaccines in 1988 that

residual endotoxin content should be tested for by means of the LAL test (Limulus amoebocyte lysate) test. First developed by Levin *et al*, it is highly sensitive and depends on clotting of the LAL in the presence of LPS (Levin & Bang, 1964).

1.3.6. Tracheal cytotoxin (TCT)

The tracheal cytotoxin is a low molecular weight glycopeptide, which is a fragment of the *Bordetella pertussis* peptidoglycan. This toxin destroys ciliated epithelial cells of the respiratory tract, probably by inhibiting DNA synthesis and inducing the production of interleukin-1 and nitric oxide (Flak & Goldman, 1996). TCT is not antigenic and has no identified role in immunity (Cookson *et al.*, 1989). It is potentially present in whole-cell vaccines as a degradation product of the peptidoglycan but has not been implicated in reactogenicity. TCT is not included in formulations of acellular vaccines (Corbel & Xing, 2004).

1.3.7. Adhesins

1.3.7.1. Filamentous haemagglutinin (FHA)

FHA plays the dominant role among these attachment factors. FHA is synthesised in a 370-kDa precursor form and is then processed to yield a 220-kDa mature protein that is both anchored to the bacterial surface and secreted in large amounts into the extracellular environment (Locht *et al.*, 1993; Makhov *et al.*, 1994). The maturation process of the protein is dependent on an outer-membrane-associated accessory protein named FhaC. This protein is able to form channels (Jacob-Dubuisson *et al.*, 1999) through which FHA is believed to cross the outer membrane (Guedin *et al.*, 1998). FHA binds to ciliated respiratory epithelial cells and other host cells such as local macrophages in a galactose-dependent lectin like manner, mediated by a carbohydrate recognition domain (Prasad *et al.*, 1993). FHA also has a RGD

sequence (Arg-Gly-Asp). The RGD sequence is recognised by two integrins LRI (leucocyte response integrin) and CR3 (complement receptor 3) on the surface of macrophages (Relman *et al.*, 1990).

FHA was found to interfere with phagocytosis by neutrophils. FHA is involved in the attachment of the bacteria to white blood cells, however attachment via opsonizing antibody is essential for internalization of the bacteria (Weingart & Weiss, 2000). In addition, Abramson *et al* from an *in vitro* study have indicated that secreted and cell-associated FHA elicit proinflammatory and proapoptotic responses in human monocyte-like cells and bronchial epithelial cells (Abramson *et al.*, 2001).

Based on the laboratory study of *B. pertussis* protective antigens since the early 1970s, FHA is one of the major components, along with detoxified PT, of acellular pertussis vaccines (Sato & Arai, 1972; Sato *et al.*, 1973; 1974). Clinical studies have indicated that vaccines containing PT and FHA are slightly more effective than those with PT alone. FHA has not been directly associated with toxicity (*Ad hoc* group of study of pertussis vaccines, 1988).

1.3.7.2. Fimbriae (Fim)

Bordetella pertussis produces fimbriae, also called agglutinogens, since they determine the serotype of the strain. Three serotypes are known: 1, 2 and 3. The most important are serotypes 2 and 3 conferred by Fim2 and Fim3. Fimbriae are composed of two subunits, the major fimbrial subunit, i.e. Fim 2 or 3, and each is terminated by a 4 kDa protein, Fim D, which binds to the integrin VLA5 of macrophages (Hazenbos *et al.*, 1995) and to sulphated sugars (Geuijen *et al.*, 1997) which are ubiquitously present in the respiratory tract. Binding of Fim D to VLA-5 activates CR3, the receptor for FHA, which thereby assures cooperativity between fimbrial and FHA binding. Recent studies using epithelial cell lines derived from the

human respiratory tract have indicated that fimbriae play a role in infection of the laryngeal mucosa, whereas FHA is important for colonisation of the entire respiratory tract (van den Berg *et al.*, 1999). Fim 2 and Fim 3 are regarded as important immunogens of *B. pertussis*, responsible for inducing the serotype-specific immunity observed in epidemiological studies on whole-cell vaccines (Preston, 1963). They stimulate protective immunity in animal models and are required component of whole-cell vaccines and some acellular vaccines. They are not known to possess toxic activity.

1.3.7.3. Pertactin (PRN) and tracheal colonization factor (TCF)

These two adhesins are described as autotransporters (Finn & Stevens, 1995). They are produced as precursor proteins but their biogenesis does not require accessory protein activity. Their extracellular location is mediated by their own carboxyl-terminal region, which most likely forms a channel in the outer membrane through which the amino-terminal moiety is translocated. TCF has been reported to feature substantial polymorphism, especially in one of its immunodominant regions (Finn & Stevens, 1995;Luker *et al.*, 1995). Both proteins contain proline-rich regions and RGD sequences thought to be involved in the adherence of the bacteria to the host tissues (Finn & Amsbaugh, 1998). Both proteins are considered to play a major role in the colonization of the respiratory tract by *B. pertussis* (van den Berg *et al.*, 1999). They are not known to exert toxic activity at concentrations produced *in vivo*. PRN is used as vaccine component.

1.3.7.4. Other *bvg*-regulated virulence determinants

Two additional autotransporters have been characterized. The first one is called BrkA (Shannon & Fernandez, 1999). The protein contains two RGD sequences and two

potential sites for binding to sulphated glycoconjugates. In addition, BrkA inhibits the classical pathway of complement activation and prevents accumulation of deposited C4 (Barnes & Weiss, 2001).

The second autotransporter is Vag8. It is a 91kDa protein homologous to PRN, TCF and BrkA and also contains an RGD site. However, it has been reported that a mutant strain of *B. pertussis* defective in Vag8 showed similar patterns with the parent strain in terms of colonisation and persisting in the lungs of mice after aerosol challenge (Finn & Amsbaugh, 1998).

B. pertussis has been reported to be a capsulated bacterium. Its genome contains a complete operon for capsule biosynthesis. However the capsule cannot be observed under standard laboratory conditions and the presence of $MgSO_4$ seems to be required. Taking this information together, it is likely that capsule biogenesis is dependent on environmental conditions and is regulated by the *bvg* genes (Antoine *et al.*, 2000). The capsule could play a major role in the early stage of infection by improving the adhesion of the bacteria to the respiratory mucosa or by helping them to avoid the innate immune response (opsonization or phagocytosis).

1.4. Immunology

The immune system is composed of two parts: innate and adaptive. Innate immunity serves as the first line of defence but lacks the ability to recognize certain pathogens and to provide the specific protective immunity that prevents re-infection. Adaptive immunity is based on the clonal selection of lymphocytes bearing highly diverse antigen-specific receptors, which allows the immune system to recognize any foreign antigen. In the adaptive immune response, antigen-specific lymphocytes proliferate and differentiate into effector cells that eliminate pathogens. It also generated

numbers of differentiated memory lymphocytes through clonal section and this allows a more rapid and effective response upon re-infection.

1.4.1. Innate immunity

1.4.1.1. The surface barrier.

Some of the defence mechanisms at the body surfaces include:

- The skin can not be penetrated by organisms unless it already has an opening (i.e. wound). Bacterial growth is generally inhibited in the surface of the skin by the presence of acidic secretions.
- The hair follicles are also involved in growth inhibition of pathogens by the secretion of sebum.
- In addition, saliva, tears contain lysozyme that destroys some Gram-positive bacteria.
- The stomach is an obstacle as its mucosa secretes hydrochloric acid and protein-digesting enzymes that kill many pathogens.
- Mechanically the pathogens are expelled from the lungs by ciliary action, coughing and sneezing.

1.4.1.2. Inflammation

Inflammation can be caused by microbial infections, the principle effects include: redness, heat, swelling and pain. The outcome of inflammation is the release of histamine by mast cells and chemotaxins by damaged cells.

1.4.1.3. Polymorphonuclear neutrophils

These are phagocytes that provide the major defence against bacteria and are the first at the site of infection, followed by the wandering macrophages about three hours later.

1.4.1.4. Macrophages

Macrophages are phagocytic cells that are attracted to the site of infection by chemokines and cytokines. Macrophages belong to the cells called antigen presenting cells (APCs) because, once a macrophage phagocytises a foreign body, it places on its surface epitopes that activate other immune cells. Each of the cells in the innate immune system binds to antigen using pattern-recognition receptors. These receptors are encoded in the germ line of each person. This immunity is passed from generation to generation.

1.4.1.5. Natural killer cells

Natural killer cells move in the blood and lymph to lyse cancer cells and virus-infected body cells. They are large granular lymphocytes that attach to the glycoproteins on the surfaces of infected cells and kill them.

1.4.1.6. Complement

The complement system is a set of plasma proteins that act together to attack extracellular forms of pathogens. Complement can be activated spontaneously on the surface of certain pathogens (by the alternative pathway) or by antibody binding to the pathogen (classical pathway). The pathogen becomes coated with complement proteins that facilitate pathogen removal by phagocytes and or by killing the pathogen by complement-mediated lysis.

1.4.1.7. Dendritic cells

The dendritic cells are mostly found in the skin and mucosal epithelium, where they are referred to as Langerhan's cells. Unlike macrophages, dendritic cells can also recognize viral particles as non-self. In addition, they can present antigens via both MHC I and MHC II, and can thus activate both $CD8^+$ and $CD4^+$ T-cells, directly.

1.4.2. Adaptive or acquired immunity

The adaptive immune system allows recognition of a wide range of different pathogens and forms a protective memory response. The cells of the adaptive immune system that specifically recognise different antigens from pathogens are the B and T lymphocytes. Antigens are molecules that induce an immune response through the activation of antigen-specific lymphocytes. The specificity of antigen recognition is determined by the antigen receptors on B and T lymphocytes.

1.4.2.1. Cell-mediated immunity

T-lymphocytes recognize specifically the antigen presented on the surface of the APCs cells (that also has "self-antigens"). The T-lymphocytes are then activated and become differentiated into: Cytotoxic or killer T cells ($CD8^+$) or Helper T cells ($CD4^+$) or memory T cells or suppressor T cells. Memory T cells, on encounter with antigen, synthesise cytokines and differentiate into cytotoxic T cells. Migrating through non-lymphoid tissue, effector memory cells are positioned to provide an immediate response following secondary contact with antigen. Suppressor T cells suppress B cell antibody production and cytotoxic and helper T cell activity. Cytotoxic (killer) T cells eliminate host cells bearing foreign antigen (e.g., host cells invaded by viruses and cancer cells)

T-helper cells play a major role in controlling infections. These cells can be divided into terminally differentiated T helper 1 (Th1), or T helper 2 (Th2) cells, depending on their ability to secrete cytokines. Th1 cells secrete INF-γ, TNF-β and to some extent IL-2. Th1 cells are implicated in cell-mediated immunity and inflammation by stimulating macrophages and recruiting leukocytes. On the other hand, Th2 cells secrete IL-4, IL-5, IL-10 and IL-13, activate eosinophils and mast cells, induce the production of IgE, and participate in allergic reactions. In addition Th2 cells stimulate

B lymphocytes and induce antibody-mediated immunity (Lloyd *et al.*, 2000; Wang *et al.*, 2004).

1.4.2.2. Humoral immunity

An immunocompetent but immature B-lymphocyte is stimulated to maturity when an antigen binds to its surface receptors and there is a T helper cell nearby to release cytokines. This primes the B cell and it undergoes clonal selection. Most of the B cells become plasma cells. Plasma cells produce highly specific antibodies. The other B cells become long-lived memory cells. The antibodies may inactivate pathogens by, (a) complement fixation (complement proteins attach to pathogen membrane and cause pore formation to induce cell lysis), (b) neutralization (binding to specifics sites to prevent attachment to host tissues), (c) agglutination (clumping), (d) precipitation (forcing insolubility). (Janeway and Travers, 1997)

1.4.3. Immunity to *B. pertussis*

Acquired immunity against *B. pertussis* develops after natural infection and confers relatively long-lived protection against subsequent infection. Immunization with whole-cell vaccines (WCV) and acellular vaccines (ACV) also protect against the disease. The identification of immunological correlates of protection against *B. pertussis* has been a major goal of many clinical studies on pertussis. However, it has not been possible to correlate protection with a quantifiable immune response against a single protective antigen. Indeed *B. pertussis* produces a large range of toxins and adhesins. These contribute to pathogenesis and many are involved in immune protection (Figure 2) or immune subversion.

PT is thought to play an important role in immune subversion by inhibiting the chemotaxis of innate immune response cells : natural killer cells, macrophages and neutrophils (Spangrude *et al.*, 1985; Allavena *et al.*, 1994; Schorr *et al.*, 1999). TCT

has also been reported to act as an antichemotactic factor on neutrophils (Torre *et al.*, 1999). ACT is also involved in the subversion of the immune response. ACT is known to induce apoptosis of monocytes (Gueirard *et al.*, 1998), to inhibit intracellular killing in macrophages which allows the intracellular survival of bacteria (Masure, 1993) and to inhibit phagocytosis by neutrophils (Weingart & Weiss, 2000). In addition, adhesins are involved in the survival of the bacteria in the respiratory tract by mediating adherence to ciliated epithelial cells, macrophages and neutrophils. Furthermore, following invasion of the respiratory tract, *B. pertussis* not only binds to epithelial cells and multiplies extracellularly but is also taken up by, and survives within, macrophages and other cell types *in vitro*, providing indirect evidence of a role for cell-mediated as well as humoral immunity in protection (Mills, 2001).

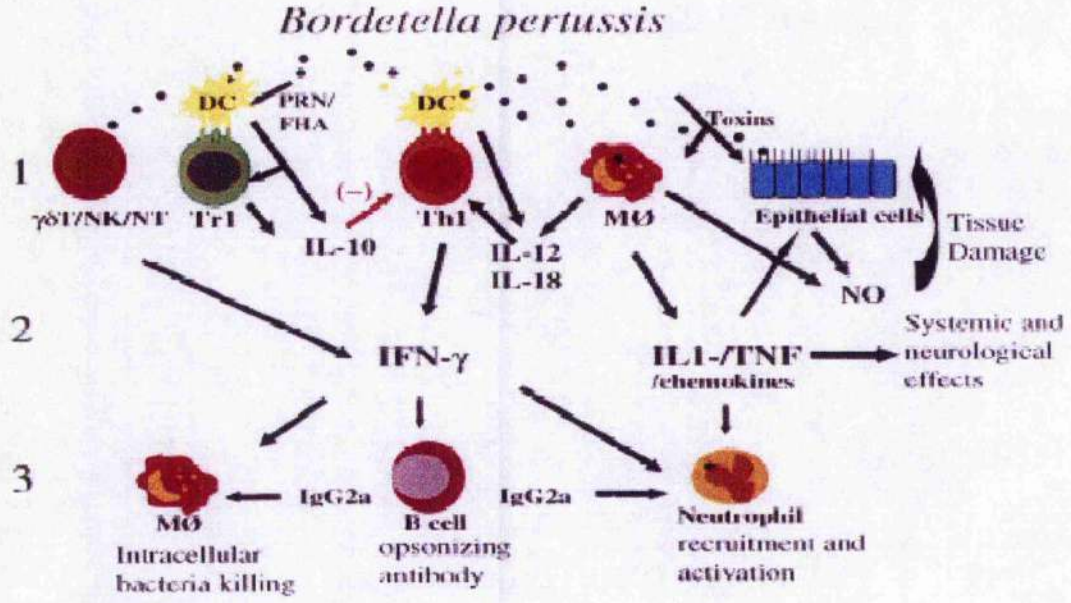


Figure 2. Immunity to *Bordetella pertussis* in a naïve host

Immunity to *B. pertussis* in a naïve host: evidence from the murine respiratory challenge model. (1) recognition of bacterial components by cells of the innate and acquired immune system, (2) production of soluble mediators and (3) recruitment and activation of effector cells and molecules. When *B. pertussis* enters in the respiratory tract, the bacteria bind to ciliated epithelial cells (Bassinet *et al.*, 2000), but are also recognised and taken up by cells of the innate immune systems, such macrophages (MΦ)(Friedman *et al.*, 1992), dendritic cells (DCs), T cells, natural killer (NK) or natural T cells (NT) (Mielcarek *et al.*, 1998). DCs process and present bacterial antigens to T cells. The production of IL-12 and IL-18 by innate cells results in the polarization of the T-cell response to the Th1 subtype. However, early in infection the local Th1 response is suppressed due to the effects of IL-10 secreted by antigen-stimulated Tr1 cells or by FHA-stimulated macrophages and DCs (McGuirk & Mills, 2000b). NO and the pro-inflammatory cytokines, IL-1β and TNF-α induced by bacterial toxins, especially LPS (Loscher *et al.*, 2000), TCT (Heiss *et al.*, 1993) and PT (Torre *et al.*, 1996;Ryan *et al.*, 1998), as well as contributing to bacterial elimination, also mediate local lung pathology and are responsible for many of the systemic and neurological consequences of the infection. IFN-γ secreted early in infection by cells of the innate immune system, and later in infection by Th1 cells, stimulates recruitment and activation of macrophages and neutrophils and provides help for B cells to secrete opsonizing and complement-fixing antibody (IgG2a in the mouse). Opsonized or non-opsonized bacteria are taken up by neutrophils and macrophages which are killed by NO or reactive oxygen intermediates (Mills, 2001).

1.5. Vaccines

Pertussis (whooping cough) is a highly contagious disease caused by *B. pertussis*. Worldwide, this bacterial agent causes some 20–40 million cases of pertussis and an estimated 200 000–400 000 fatalities each year. Although pertussis may occur at any age, most cases of serious disease and the majority of fatalities are observed in early

infancy. Vaccines are the most rational approach to pertussis control. For several decades, inactivated whole-cell vaccines (WCV) have been part of national childhood vaccination programmes, dramatically reducing the considerable public health impact of pertussis. These vaccines are currently being produced in over 40 countries, including many developing countries. Currently, worldwide pertussis vaccination coverage is about 80%. Frequent (but usually mild) adverse reactions and a fear of rare but serious acute or chronic neurological events associated with WCV vaccination have prompted the development of a new generation of pertussis vaccines, the acellular (ACV) vaccines. However, despite thorough investigations, the link suspected between WCV vaccines and rare cases of permanent neurological damage has not been confirmed. The ACV vaccines, which contain one to five different components of *B. pertussis*, have proved to be efficacious, although more expensive, and to compare favourably with WCV vaccines in terms of common adverse effects. They are now licensed in several countries. At their most effective, ACV and WCV vaccines share similar efficacies. Both WCV and ACV are usually administered in combination with diphtheria and tetanus toxoids (DTwP or DTaP)(Galaska, 1993).

1.5.1. Whole-cell vaccines (WCV)

B. pertussis is grown under conditions that favour expression of the virulent Phase I phenotype. Strains are selected to cover agglutinogen serotypes 1, 2 and 3. In some cases, a single strain of serotype 1, 2 or 3 is employed but often one or more strains are used. The bacterial cells are harvested and inactivated under conditions that preserve their protective antigens in antigenic form but eliminate excessive toxicity. The conditions of inactivation and detoxification vary according of manufacturers.

Some use thiomersal in combination with mild heat treatment, others use mild heat treatment alone, or formaldehyde treatment in order to produce an inactivated bulk. The bulk is further diluted and blended with aluminium-adsorbed diphtheria and tetanus toxoids.

Potency and toxicity testing is usually performed at the bulk stage. The only internationally accepted potency assay is the mouse intracerebral challenge test. Safety testing is focused on the mouse weight gain test (MWGT)(Requirements for diphtheria, tetanus, pertussis and combined vaccines, 1990b).

1.5.2. Acellular vaccines (ACV)

The first ACV vaccines were developed in Japan, where such vaccines were licensed for immunization of children aged two years or more in 1981, and for infants from the age of three months in 1989. The first DTaP combination was licensed in the United States in 1991, at first as an alternative to DTwP boosters in children who had received their basic DTwP series. Vaccination with ACV, starting at the age of two months, is now included in routine childhood vaccination programmes in several countries. It is not known whether the duration of protection with ACV vaccines is the same as with WCV vaccines.

Acellular vaccines are, by definition, cell-free preparations. Two approaches have been used for the processing of the starting material: copurification and individual purification of components. In the first approach, vaccine components have been co-purified in order to concentrate some components and remove others such as LPS. The example of this is the Takeda (T) vaccines. T-type vaccines contain more FHA and less PT. T-type vaccines contain agglutinogens (fimbriae) and pertactin/or other unidentified component(s), in addition to PT and FHA (Kamiya & Nii, 1988).

The second approach is to purify each component individually. The purified components, after detoxification, are blended to produce acellular vaccine bulk. The acellular vaccines are usually combined with vaccines against diphtheria and tetanus as DTaP vaccines. The Pa part of DTaP-vaccines is usually described by the number of characterized protein antigens. One component vaccines (Pa1) contain only chemically detoxified or genetically-detoxified PT (Robbins *et al.*, 1993). Two-component vaccines (Pa2) contain PT and FHA (Leininger *et al.*, 1997). Three-component vaccines (Pa3) contain PT, FHA, and PRN (Shahin *et al.*, 1990). And the four to five-component vaccines (Pa4 and Pa5) contain PT, FHA, PRN and one or two fimbrial agglutinogens Fim2 and Fim3 (Geuijen *et al.*, 1996). The degree of purification can vary widely depending on the process followed. Due to the chemical toxoiding process, acellular vaccines do not have a completely defined chemical composition. This means that biological testing is still required to monitor batch-to-batch variation. Safety testing is directed towards monitoring specific components, especially PT and LOS (Corbel & Xing, 2004).

1.5.3. Vaccine-induced immunities.

Whole-cell vaccine contains endotoxin (LOS), which activates IL-1β, TNF-α, IL-12 and IL-18 production by macrophages and other cells of the innate immune system (Loscher *et al.*, 2000). These pro-inflammatory cytokines regulate the selective induction of Th1 cells from the precursor T cell (Thp). Cytokines secreted by Th1 cells (INF-γ), provide help for opsonizing antibody production and activate macrophages and neutrophils to take up and kill intracellular bacteria.

Following priming with acellular cell vaccine, there is a less significant increase in the numbers of neutrophils or T-cells in the lung after challenge (McGuirk & Mills, 2000a). This is explained by the composition of the acellular vaccine that is devoid of

bacterial toxins that stimulate IL-12 and IL-18 (LOS). However, acellular vaccine include components such as FHA which stimulate IL-10 production and consequentiy have anti-inflammatory activity and induce Th2 cells (McGuirk & Mills, 2000b).Th2 cells provide help for B cells to secrete IgE and murine IgG1, and IgG3 antibodies which neutralize toxins and prevent adherence of bacteria in the respiratory tract (Mills, 2001).

1.5.4. Comparison of whole-cell and acellular vaccines.

Whole-cell vaccines are considerably less costly than the acellular vaccines. Therefore, in most countries, WCV remain the appropriate choice for public health immunization programmes. While in terms of severe adverse effects ACV appears to be safe, mild to moderate adverse reactions are also less commonly associated with ACV than with WCV. Similar high efficacy levels are obtained with the best ACV and WCV, but the level of efficacy may vary considerably between the vaccines within these two groups. Reliable comparisons of different ACV and WCV, or between ACV and WCV, are possible only in studies that are carefully designed for that purpose. So far, no trial has had the optimal design to adequately compare different candidate antigens, and the choice and number of antigen components of the ideal ACV is still debated. WHO endorses the use of ACV vaccines of documented quality in countries where pertussis vaccination is not widely accepted because of the reactogenicity of WCV. The main impediments to wider use of the ACV are their high price and concern about their duration of protection. If these issues can be satisfactorily resolved, widespread use of this product will be encouraged in the long term. Areas in need of further research include the duration of protection following primary immunization with WCV or ACV, interference between ACV and other vaccines when

used in combination, ability of ACV to induce a side effect, and the epidemiology of pertussis in the adult population (Immunization, vaccines and biologicals: Pertussis vaccine, 2003).

1.5.5 The re-emergence of Pertussis

Studies based on the polymorphisms of genes encoding WCV and ACV components found in isolates of *B. pertussis* in some countries (The Netherlands, France) suggest that, within 10-20 years after the introduction of mass vaccination, an adaptive response occurred, consisting in clonal expansion of *B. pertussis* strains, that expressed a PT variant distinct from the PT in vaccine strains. (van Loo *et al.*, 2002a-b, Weber *et al.*, 2001). Such adaptation may have allowed *B. pertussis* to remain endemic despite widespread vaccination and may have contributed to the re-emergence of Pertussis in some countries (Mooi *et al.*, 2001). However, van Amersfoorth *et al.* (2005) characterised 102 *B. pertussis* isolates from the period 1998 to 2001 from five European countries (Finland, Sweden, Germany, The Netherlands, and France). The isolates were analysed by typing based on variable number of tandem repeats (VNTR); by sequencing of the polymorphic genes encoding the surface proteins pertussis toxin S1 and S3, pertactin, tracheal colonization factor and by fimbrial serotyping. In this study, no relation was detected between the strain characteristics, including the gene polymorphisms, and vaccination programmes. Despite these findings the polymorphisms may have implications for the efficacy of both whole-cell and especially acellular vaccines with their more limited antigenic repertoire. There is therefore a need for continued surveillance and typing of circulating strains and there may be a future need to adjust

the composition of these vaccines to contain several pertactin and PT types (Mooi *et al*, 1998).

1.5.6. Potency tests

The intracerebral mouse protection (Kendrick) test is the official potency assay for whole-cell pertussis vaccines and is the only test which has shown a correlation with protection in children (Kendrick, 1947). Groups of mice are immunized (intraperitoneally) with serial dilutions (2.5, 0.5 and 0.1 IU/ml) of the reference vaccine and the test vaccine. At 14-17 days after immunization, mice are challenged intracerebrally with a specific strain of *B. pertussis* (18-323). The mice are observed for lethal effects over the next 14 days. The potency is estimated in terms of International Units by a parallel line assay (Requirements for diphtheria, tetanus, pertussis and combined vaccines,WHO Technical Reprot Series No. 800, 1990a). As the conventional intracerebral challenge test proved unsuitable for determining potency of acellular vaccines, a modification of this was developed in Japan. It increased from 2 weeks to 3 weeks the period between immunization and challenge and also a special mouse strain was preferred (Sato, 1996). For several reasons, including animal welfare, the use of a challenge route unrelated to the natural mode of infection, technical difficulty and reproducibility, it has been suggested that an alternative test needs to be developed (Corbel & Xing, 2004).

The use of intranasal or aerosol challenge to produce murine respiratory tract infection with *B. pertussis* has considerable potential value for evaluating the protective activity of acellular pertussis vaccines. The end point can be measured by comparison of the response to the test vaccine, in terms of bacterial colonisation of

the mouse lungs, with that to a vaccine of known clinical efficacy or an appropriate reference preparation. Although overt symptomatic disease is not elicited in these models, several characteristics of the human infection are reproduced, such as multiplication and clearance of the bacteria, limitation of infection to the respiratory tract, increased severity of infection in young animals, and various systemic physiological changes. Recent studies have shown that this may also be a useful model for the preclinical assessment of acellular pertussis vaccine (Guiso *et al.*,1999; Xing *et al*, 2002a; Corbel & Xing, 2004).

1.5.7. Toxicity tests

1.5.7.1. Whole-cell vaccines

Currently, for control of whole-cell pertussis vaccines, the mouse weight gain test (MWGT) is the only test specified by WHO, European Pharmacopoeia (EP) and US requirements. It can be considered as a general, non-specific test measuring overall toxicity, since a number of *B. pertussis* toxins may affect the weight gain of mice. Correlation of the results of the MWGT with adverse reactions in children has been reported (Cohen, 1969; Perkins, 1970). The mechanism of the MWGT is unclear. Endotoxin is the main toxic component detected by the test but PT tends to counteract the endotoxin-induced weight loss by its insulin-releasing effect (Gupta *et al.*, 1988).

A volume (0.5 ml) of the test vaccine is injected intraperitoneally into a group of 10 mice. The groups are observed and body weights are recorded on days 0, 3 and 7. By comparison with a control group, a vaccine is considered safe when: 1. the total weight of the mice from the vaccine group at 3 days after treatment is not less than it was at day 0; 2. at the end of 7 days, the average weight gain per mouse in the test

group is not less than 60% of the control; 3. not more than 5% of the total numbers of injected mice die (van Straaten-van de Kappelle *et al.*, 1997).

1.5.7.2. Pertussis toxin

PT in its detoxified form is an important component of both whole-cell and acellular pertussis vaccines. Safety tests are thus required by regulatory authorities to assure the absence of significant residual active toxin or reversion of detoxified toxoid to its active form in pertussis vaccines. The histamine-sensitising (HIST) assay and Chinese hamster ovary (CHO) cell assay are used widely for this purpose to monitor the active PT content of pertussis vaccines (Xing *et al.*, 2002b).

1.5.7.2.1. Histamine sensitisation test (HIST)

PT toxoid is an important component of whole-cell and acellular vaccines and regulatory authorities require safety testing of pertussis vaccines in order to confirm absence of residual pertussis toxin toxicity. The histamine sensitisation test is the only test considered by the regulatory authorities to be suitable for this purpose (Guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines.1998; Diphtheria, tetanus, pertussis vaccine.2002b). In the histamine sensitisation test (HIST) groups of mice are injected intraperitoneally with doses of the test vaccine. Four to five days after injection, animals are challenged intraperitoneally with a histamine solution (2mg/0.5ml/dose) and the number of mice dying within 24h is recorded. HIST is a lethal test and large variations in test performance have been observed and demonstrated to be dependent upon such variables as: mouse strain, number, age, injection route and challenge route. The HIST is difficult to standardize, its precise mechanism is unknown and it must be regarded as a priority for replacement (van Straaten-van de Kappelle *et al.*, 1997).

1.5.7.2.2. Role of PT in the histamine sensitisation test

The exact mechanism of the HIST test is still unknown, but studies have shown that vaccination of rats with biologically-active PT decreases diastolic blood pressure and enhances histamine-induced decrease in mean arterial blood pressure. This histamine sensitisation mainly involved histamine H1 receptors (Vleeming *et al.*, 2000). In addition, van meijeren *et al.* (2004a; 2004b) found that PT pre-treatment of male Wistar rats reduced maximal noradrenalin- or KCl- induced contraction of isolated small mesenteric resistance arteries, decrease sensitivity to noradrenaline of isolated rat small mesenteric resistance arteries and did not affect histamine- or acetylcholine-induced relaxation. They concluded that PT-induced histamine sensitisation is caused by an interference of PT with the contractile mechanisms of vascular smooth muscle of resistance arteries which indicates only an indirect role for histamine in the histamine sensitisation test.

1.5.7.2.3. CHO cell assay

The CHO cell clustering test is prescribed in the WHO guidelines as a sensitive and useful technique for evaluating the detoxification of PT in the process of acellular pertussis vaccine production (Arciniega *et al.*, 1998). In this test, the CHO cells are treated with a PT reference preparation or vaccine dilutions. After incubation, the degree of clustering of the cells is observed and scored under an inverted microscope. The highest dilution of the test vaccine showing total cell clustering represents the titre. Unfortunately, the CHO-cell assay is not suitable for testing final vaccine formulations as the presence of adjuvant causes the death of the CHO cells (Gillenius *et al.*, 1985).

1.5.7.3. LPS

LPS is considered to be the major component of the whole-cell vaccine that induces vaccine reactogenesis. LPS does not affect the protective efficacy of the vaccine so the component has not been included in acellular vaccines formulations (Chaby, 1988). However, some types of acellular vaccine (e.g. the co-purified type) could be contaminated by LPS during the purification processes. Therefore, both whole-cell vaccines and acellular vaccines must be tested for the presence of LPS. For this purpose, the LAL assay, developed by Levin and Bang, is the most appropriate test available.

Frederick Bang observed that bacteria caused intravascular coagulation in the American horseshoe crab, *Limulus polyphemus*. In collaboration, Levin and Bang (1964) found that the agent responsible for the clotting phenomenon resided in the crab's amoebocytes, or circulating blood cells, and that pyrogen (bacterial endotoxin) triggered the turbidity and gel-forming reaction enzymatically. The LAL test permits a precise measurement of endotoxin content of a given sample. In this test, a volume of serial dilutions of the test specimen (1/10 to 1/100,000) and lysate (10 µl) are incubated in pyrogen free-tubes. The definition of end point is the formation of a firm gel that can be detected by eye or by spectrophotometric measurement of a change in a chromogenic peptide substrate. The LAL test is used widely as a simple and highly sensitive *in vitro* method for the detection of endotoxin (Nakagawa *et al.*, 2002).

1.6. Cell lines for *in vitro* studies of the molecular mechanisms of whooping cough

1.6.1. Bronchial epithelial cell lines BEAS-2B and NL20

Respiratory ciliated epithelium is the primary site of infection of *B. pertussis*. The use of bronchial epithelial cell lines as models for study has improved our understanding of the biology of disease.

BEAS-2B, a bronchial epithelial cell line, has been used in various studies of *B. pertussis*. Ishibashi *et al* have demonstrated that the FHA of *B. pertussis* up-regulates the expression of epithelial ICAM-1 in these cells and that PT impairs this response (Ishibashi & Nishikawa, 2002). The same team further demonstrated that FHA binds to the integrin VL5 of BEAS-2B cells and induces an RGD-dependent NF-kappa B activation, thus leading to the up-regulation of epithelial ICAM-1 expression. They also showed that a PT-sensitive G protein may be involved in this signalling pathway (Ishibashi & Nishikawa, 2003). Taking this information together, it is possible that PT could be involved in the delay of the recruitment and accumulation of inflammatory cells at the site of infection. Another study demonstrated that migration of peripheral blood mononuclear cells across a monolayer of BEAS-2B cells was significantly inhibited by PT. This result implicates a G protein signalling event as an important mediator of lymphocyte/monocyte transepithelial migration (Miller & Butcher, 1998). It has also been reported that PT can block activation-dependent binding of lymphocytes to endothelium *in vivo* (Bargatze & Butcher, 1993). Belcher *et al* used microarray technology to investigate the transcriptional response of the BEAS-2B cells in response to *B. pertussis* treatment. The early transcriptional response to this pathogen was dominated by altered expression of cytokines, DNA

binding proteins and NFκB-regulated genes. Also the bacteria induced mucin gene transcription by the epithelial cells. The pathogen counters the innate defence of the respiratory tract by using mucin as a binding substrate (Belcher *et al.*, 2000).

NL20 is an immortalised, human bronchial epithelial cell line (Schiller & Bittner, 1995). It is an attached cell type and requires growth medium additives to optimise its cell proliferation. These additives are insulin, epidermal growth factor, hydrocortisone transferrin and non-essential amino acids.

1.6.2. Human umbilical endothelial cell (HUVEC)

Respiratory tract infections caused by *B. pertussis* are occasionally accompanied by severe neurologic disorders and encephalopathies (Mortimer & Edward, 1998). Encephalopathy has also been reported in children receiving pertussis vaccination. PT (or PTd) is a component of all whole- cell and acellular vaccines (Miller, 1993). Its structure and function have been widely investigated but its role in the disease and in the toxicity of the vaccine is still not understood. In the pathogenesis of pertussis-related neurological disorders, an important effect might be on the integrity of the cerebral barrier. The *Plexus chorioideus* (Pannese *et al.*, 1994) is composed of capillaries. They are overlain by a neatly cuboidal epithelial covering and protrude into the lumen of the ventricles in the brain. The cells produce cerebrospinal fluid that fills the ventricles of the brain and the lumen of the spinal cord (Pannese *et al.*, 1994). Bruckener *et al.* (2003) recently investigated, in the *Plexus chorioideus* model, the role of PT on the epithelial barrier and on the endothelial barrier. They concluded that PT does not affect the epithelial barrier but it does modify the morphology of the endothelial cells. The permeabilisation of cerebral endothelial monolayers by PT proceeds via the phosphokinase C pathway. In addition, Adamson *et al.* (2002) study showed that treatment of brain endothelial cell (EC) monolayers with PT resulted in

ADP-ribosylation of G-protein α subunits and inhibition (>80%) of lymphocyte migration without affecting lymphocyte adhesion. Thus, with regard to the induction of encephalopathies as a potential consequence of pertussis infection, which has recently been discussed by Donnelly *et al.* (2001), PT might exert a dual effect in permeabilizing cerebral endothelial barriers mediated by the activity of the ADP-ribosyltransferase and, by contrast, mediating an anti-inflammatory effect by competitively interfering with leukocyte recruitment (Rozdzinski *et al.*, 1993).

In order to investigate the eventual binding or toxic effect of PT on endothelial cells in this study, HUVEC primary cells (Cambrex CC-2519) will be used as cellular model.

1.7. Microarrays

First described in 1995 (Schena *et al.*, 1995), DNA microarrays are a powerful tool that allows the simultaneous analysis of a large number of nucleic acid hybridization experiments in a rapid and efficient fashion. DNA microarrays are based on the reverse of the Southern blot technique. Gene-specific oligonucleotide probes are immobilized on a membrane or a glass slide and then hybridized against the labelled target population of cDNAs (Kurian *et al.*, 1999)

The applications of DNA microarray technology include the comprehensive analysis of multiple gene mutations and expressed sequences with regard to drug design (pharmacogenetics), drug side effects (toxicogenomics) and host-pathogen interactions.

1.7.1. Advantages and limitations of microarrays

From the recent completion of the sequencing of the human genome (Rogers, 2003) and the *B. pertussis* genome (Parkhill *et al.*, 2003), microarrays would seem to provide a powerful tool to investigate the interaction of the pathogen with its host.

Established methods of studying gene expression such as Northern blotting and quantitative PCR are inherently serial, involving measuring a single gene at a time and are difficult to automate. The major advantage of DNA microarrays is that they permit simultaneous detection of expression levels for nearly every gene in an organism or cell line in one experiment. Further advantages are that microarrays are high throughput, versatile and fast.

Microarray technology should allow us to unravel the function of genomic data into fundamental biological principles and enable the design of molecularly-defined vaccines to target pathogens specific to given human genotypes, and also enable the prediction of potential immune responses to a given antigen (Dhiman *et al.*, 2001). Nevertheless, DNA microarrays are a new technology that has limitations.

The major limitation of the current technology is the shortcomings of DNA management, statistical analyses and cost (Dhiman *et al.*, 2001). Another drawback of microarray technology is that it limits the expression studies to the mRNA level. Ideally, it should be accompanied by analyses at the protein level. Proteomics focuses on different steps of the same process: the expression of genetic information into functional molecules, cells and organisms. Moreover, proteomics allows the detection of inducible modifications (phosphorylation and glycosylation) of the gene products, which are not regulated at the transcriptional level (Dhiman *et al.*, 2001).

There are major concerns regarding the sensitivity of detection of mRNA transcripts that are in low abundance and the time required for analysis and interpretation of data. The most critical concern is whether gene expression profiles for a given system are consistently reproducible from study to study and across different laboratories. An additional challenge is to define appropriate standards and controls within and between institutions (Benes & Muckenthaler, 2003).

1.7.2. Applications

1.7.2.1. Study of host-pathogen interactions

The complex interactions between a microbial pathogen and a host are the underlying basis of infectious disease. DNA microarrays exploit primary sequence data to measure simultaneously transcript levels for every gene that may play a role in the host-microbe interactions (Boldrick *et al.*, 2002; Eskra *et al.*, 2003; Galindo *et al.*, 2003; Virok *et al.*, 2003). This technology also offers the opportunity to examine the global and subtle changes in gene expression in relation to specific stimuli (Boshoff *et al.*, 2004).

Belcher *et al.* (2000) investigated the interaction of the bacterium *B. pertussis* with the bronchial epithelial cell line BEAS-2B. They studied the mRNA differential transcription of the cells cultured in presence of the bacteria or in the presence of PT by microarray technology. They concluded that the early transcriptional response to this pathogen was dominated by altered expression of cytokines, DNA-binding proteins, mucin and NF-κB regulated genes. *B. pertussis*-infected cells exhibited a transcriptional profile dominated by a proinflammatory response. A number of chemokines were up-regulated: IL-8, GRO-1, GRO-2, GRO-3, MCP-1 and SCYA3. These chemokines are neutrophil chemoattractants (Hammond *et al.*, 1995) and have been related to lymphocyte or monocyte tissue infiltration (Jinquan *et al.*, 1995). In addition, the transcription profile of the cells infected with the bacteria revealed an upregulation of genes involved in the NF-κB pathway. TNFAIP3 and, API2 are suggestive anti-apoptotic signalling molecules and TRIP, which inhibits NF-κB activation, was downregulated. Taken together, these results suggest that the bacterium might induce apoptosis of the inflammatory cells (monocytes, neutrophils

(Khelef & Guiso, 1995)) while sparing the ciliated epithelium on which it survives (Belcher *et al.*, 2000).

Microarrays can also be used to investigate the transcriptional profile modification of the host by a specific toxin, in order to elucidate the role of a specific virulence factor in pathogenesis. Belcher *et al.* (2000) used BEAS-2B cells and PT treatment to define the role of the ADP-ribosyl transferase activity of PT. Some of the up-regulated genes identified were G-protein- or cAMP-regulated, such as those encoding matrix metalloproteinase 1, basic fibroblast growth factor and the Na^+/Cl^- dependent serotonin transporter. Belcher *et al.* also concluded that PT or *B. pertussis* up-regulation of genes involved in mucin synthesis and the PT-induced alterations in VIP and serotonin signalling could lead to thicker mucus. They demonstrated that *B. pertussis* binds to mucin, suggesting that the pathogen may be manipulating the host defences to create a favourable microenvironment for itself.

Another study was carried out by Boldrick *et al.* (2002) using microarrays to investigate the transcriptional profile of human peripheral blood mononuclear cells co-cultured with live *B. pertussis*. This study demonstrated the innate immune response induced by the pathogen. They found a preponderance of immune activation genes involved in cell-cell signalling, genes whose products participate in intercellular immunoregularory signalling pathways, and other proinflammatory mediators of the immune response. The most prevalent classes among the commonly-induced genes included cytokines [interleukin (IL) 1α, IL1β, IL6, IL10, tumor necrosis factor (TNFα), granulocyte colony stimulating factor (CSF)] and chemokines [IL8, macrophages inflammatory protein (MIP)1α, MIP1β, MIP2α], as well as cell-surface receptors and ligands (CD40 and CD40 ligand, IL2 α receptor). The time of incubation of the bacteria with the cells was found to be very important

because it could lead to contradictory results. For example, genes encoding TNF, MIP1β, IL1α and IL1β were induced quickly after exposure to either live or killed bacteria. However, while the induction of these genes was sustained in PBMCs treated with killed bacteria, their transcripts rapidly diminished in the cells exposed to live *B. pertussis.* The ability of live *B. pertussis* to suppress expression of these important antimicrobial genes suggests that active mechanisms are used by the bacteria to influence the host response. PT could be responsible for the TNF-α suppression. An increase in host intracellular cAMP levels through the actions of PT and ACT is a crucial feature of *B. pertussis* virulence (Katada *et al.*, 1983). The increase of intracellular cAMP could explain the abrogation of the LPS-stimulated induction of TNF-α expression (Zidek, 1999).

The chemoattractant effect of LPS on neutrophils has been studied using microarrays. Neutrophils isolated from healthy donors were incubated for 4h in presence of LPS from *E. coli* 0111:B4. The gene expression of the cells showed that LPS treatment lead to the up-regulation of genes coding for cytokines, chemokines, cell growth and interferon response whereas genes involved in cytoskeletal regulation were predominantly repressed. These date suggest molecular mechanisms by which neutrophils respond to infection and indicate that the transcriptional potential of neutrophils is greater than previously thought (Malcolm *et al.*, 2003).

1.7.2.2. Pharmacogenetics and toxicogenomics

Microarrays are powerful tools that have at least three major applications for pharmacogenetics. First, they aid in the discovery of new drugs targets. Microarrays simultaneously compare expression of thousands of genes in normal versus diseased tissue. This allows the identification of genes that are uniquely expressed in

disease, and which are potential targets for drugs action. The same genes are, in principle, also interesting as potential early detection markers for diagnostic purposes. Secondly, microarrays are used to clarify molecular mechanisms of drug action and to predict drug efficacy and toxicity (toxicogenomics)(Newton *et al.*, 2004; Moggs *et al.*, 2004). Toxicogenomics can be defined as identification and characterization of molecular mechanisms that lead to the adverse effects of xenobiotics on gene expression. Toxicogenomics is based on the fact that most relevant toxicological outcomes originate from early changes in gene expression (Storck *et al.*, 2002).

The majority of studies are performed on rodents despite the fact that the human predictability of standard rodent tests shows only 45% concordance (Johnson *et al.*, 1990). However, primary hepatocytes are well suited for toxicogenomics studies because they display a certain level of metabolic activity and the liver is a major stage for toxic events (Waring *et al.*, 2001). In addition, the use of cell culture models reduces animal utilization and the need for the synthesis of new compounds on a large scale (Baker *et al.*, 2001). Nevertheless, there are a number of limitations using *in vitro* approaches such as the functional differences observed in primary hepatocytes relative to the intact liver, the absence of interactions with biological entities (eg organs, blood) under *in vitro* conditions, the difficulty in selecting doses and time points which are representative of an *in vivo* situation (de Longueville *et al.*, 2003).

A desired outcome of expression monitoring in toxicology would be to identify a small subset of genes *in vitro* that would correlate with toxicity *in vivo* (Cunningham *et al.*, 2000). To this end, Zhao *et al.* (2003) have carried out preliminary investigations on the gene expression changes in mouse spleen cells induced by WCV, ACV, and

individual *B. pertussis* toxins, eg. PT, detoxified PT and LPS using microarray technology. The genes that were differentially expressed after pertussis vaccine treatment could be classified into a number of functional categories. These included cytokines and their receptors, growth factors, transcription factors and other immune system proteins (e.g NO, IL-1 receptor, IL-4 and TGF-β3). A number of up-regulated genes eg. TNF-19 receptor, pre-B lymphocyte gene-1, metallothionein-1 and B-cell receptor-associated protein, could possibly be associated with the toxic mechanisms of *B. pertussis*, involving apoptosis, necrosis, inflammation, inhibition of DNA synthesis or oxidative stresses (Zhao Y, 2003).

1.8. Aims of the study

The main aim of this study was the identification of gene markers of PT toxicity using the microarray technique, in order to propose *in vitro* assays based on human cell lines as an alternative to the HIST and MWGT. The study had three main goals: 1) determination of suitable concentrations of PT for microarray experiments, 2) investigation of gene expression of HUVEC and NL20 cells after treatment with PT or detoxified PT using the microarray technique, and 3) confirmation of the expression of candidate gene using semi-quantitative RT-PCR.

2. MATERIALS AND METHODS

2.1. Biological reagents

Table 1. Biological reagents.

Preparation (and batch number)	Stock solution concentration	Composition of stock solution buffer
Pertussis toxin (PAC090)	25 µg/ml	NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.15g/L, KH_2PO_4 0.2g/L, Glycine 3.75 g/L, Lactose 3.75 g/L, Saccharose 3.75 g/L, Arginine 3.75 g/L, pH 7.8
Pertussis toxin (2133)	85 µg/ml	50% (v/v) Glycerol in distilled water
Pertussis toxin (90/518)	20 µg/ml	Trehalose 5 g/L, NaCl 0.25 g/ml,Sodium Monobasic 5 mM, Sodium Bibasic 5 mM, pH 7.6
Pertussis toxin (JNIH-5)	10 µg/ml	Glucose 30 mg/l, Lactose 30 mg/L, Sucrose 30 mg/L, Arginine 30 mg/L, pH 7.7
Chemically detoxified PT (2120)	515 µg/ml	NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.15 g/L, KH_2PO_4 0.2 g/L, Glycine 33 g/L, Lactose 33 g/L, Saccharose 33 g/L, Arginine 33 g/L, pH 7.8
LPS from *B. pertussis* W28 (89/670)	25 µg/ml	Trehalose 3 g/L
Interleukin 1 beta (86/680)	100000 IU/ml	Trehalose 3 g/L, human serum albumin 0.5 g/L, isodium phosphate buffer $5x10^2$ mol/L
Tumor necrosis factor alpha (88/786)	46500 IU/ml	Human serum albumin 3 g/L

2.2. Cell lines and culture conditions.

Jurkat cells (ECACC 88042803), a human leukaemic T cell lymphoblast cell line, were grown in RPMI media (Sigma R8758) supplemented with 5% (v/v) foetal calf serum (FCS) (Invitrogen 10108-165), 2 mM glutamine (G6392) and 1% (v/v) streptomycin- penicillin (Sigma P0781). Cells were passaged every two or three days.

SHSY5Y cells (ECACC 94030304), a human neuroblastoma cell-line, were grown in MEM/F12 1:1 (Invitrogen 31330-038) supplemented with 15% (v/v) FCS (Invitrogen 10108-165), 2 mM glutamine and 1% (v/v) streptomycin-penicillin (Sigma). Cells were passaged every three days.

NL20 cells (ATCC CRL-2503), epithelial bronchial cells immortalized with SV40 large T plasmid, were grown in DMEM/F12 media (Invitrogen) containing 2 mM glutamine, 3 g/l D-glucose, 3.5 g/L HEPES and supplemented with 5% FCS (v/v) (Invitrogen 10108-165), 10 ng/ml epidermal growth factor (Sigma E9644), 0.1 mM non-essential amino acids (Invitrogen 11140), 5 μg/ml insulin (Sigma I9278), 50 μg/ml gentamycin (Sigma G1272) and 500 nM hydrocortisone (Sigma H6909). Cells were passaged every two days.

Human Umbilical Vein Endothelial primary cells (HUVEC) (Cambrex CC-2519) were grown in endothelial cell media system (Cambrex CC-3124) supplemented with bovine brain extract with heparin, human endothelial growth factor, hydrocortisone, gentamycin, amphotericin B (Cambrex CC-3124), foetal calf serum (FCS) 10% (v/v) (Invitrogen 10108-165). Cell were used from passages 1 to 6 and grown in 1% gelatin-coated (Sigma G1393) tissue culture flasks. Cells were passaged when they reached 80% confluence.

2.3. Cytotoxicity test

Two different cytotoxicity assays were performed in this study, i.e. Alamarblue and MTT assays.

2.3.1. Alamarblue assay

Cells (Jurkat, NL20, SHSY5Y and HUVEC) at $5x10^5$ cells/ml were seeded (45 µl) in a 96-well plate. Alamarblue dye (10µl) was added to each well and the plates were incubated at 37^0C in a humidified atmosphere containing 5% v/v of CO_2. After incubation for 0, 6, 24 and 48h, cells were treated with serial dilutions of PT, detoxified PT (2120) or LPS from *B. pertussis* W28 (89/670) at concentrations ranging from 0, 0.3, 0.6, 1.25, 2.5, 5 to 10 µg/ml. Toxin diluents were included as controls throughout the experiments. Cells incubated with medium and FCS alone were used as positive controls. The cells were again incubated at 37^0C in a humidified atmosphere containing 5% CO_2. For each cell line the experiment was carried out on three different batches of cells. In each experiment the concentrations of toxin were investigated in triplicate.

After treatment for 0, 6, 24 and 48 h, the cells were examined for morphological changes and the percentages of cell viability were calculated as follows. Alamarblue compound is a reduction indicator modified by mitochondrial activity to a compound that can be detected using a spectrophotometer. Alamarblue in its reduced form is detected at two wavelengths 570 nm (λ1) and 600 nm (λ2). Alamarblue dye in a testing sample was performed according to manufacturer formula (Alamarblue^{tm} assay U.S Patent No 5, 501, 959):

The calculation of the % Reduced is as follow:

λ_1 = 570 nm

λ_2 = 600 nm

$$\% \text{ Reduced} = \frac{(\varepsilon_{ox}\,\lambda_2)\,(A\,\lambda_1) - (\varepsilon_{ox}\,\lambda_1)\,(A\,\lambda_2)}{(\varepsilon_{red}\,\lambda_1)\,(A'\,\lambda_2) - (\varepsilon_{red}\,\lambda_2)\,(A'\,\lambda_1)} \times 100$$

Where:

$(\varepsilon_{red}\,\lambda_1)$ = 155,677 (Molar extinction coefficient of reduced alamarblue at 570 nm)

$(\varepsilon_{red}\,\lambda_2)$ = 14,652 (Molar extinction coefficient of reduced alamarblue at 600 nm)

$(\varepsilon_{ox}\,\lambda_1)$ = 80,586 (Molar extinction coefficient of oxidized alamarblue at 570 nm)

$(\varepsilon_{ox}\,\lambda_2)$ = 117,216 (Molar extinction coefficient of oxidized alamarblue at 600 nm)

$(A\,\lambda_1)$ = Absorbance of test wells at 570 nm

$(A\,\lambda_2)$ = Absorbance of test wells at 600 nm

$(A'\,\lambda_1)$ = Absorbance of negative control wells which contain medium plus alamarblue but to which no cells have been added at 570 nm

$(A'\,\lambda_2)$ = Absorbance of negative control wells which contain medium plus alamarblue but to which no cells have been added at 600 nm

The calculation of the % of cell viability is as follow:

$$\% \text{ cell viability} = \frac{\text{Mean of \% reduced (test)}}{\text{Mean of \% reduced (control positive for growth)}} \times 100$$

2.3.2. MTT assay

The Cell titer96® Non radioactive cell proliferation assay (Promega G4000) was used. The assay is based on the cellular conversion of a tetrazolium salt (MTT for (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into a formazan product, detected using a spectrophotometer.

Cells at $5x10^5$ cells/ml were treated in a 96-well plate with toxin solutions at concentrations ranging from 0 to 10 µg/ml and or with their associated diluents (final volume 90 µl) and the plates were incubated at 37^0C in a humidified atmosphere containing 5% CO_2 for 0, 0.5, 3, 6 and 24h. MTT dye (10µl) was then added to each well and the cells were incubated for an additional 1 hour. The solubilisation solution was then added to the reaction and the plates incubated for 4 h at 37^0C. After incubation, morphological changes were observed under a microscope (Olympus XS51) and optical density (570 nm) was recorded using a Multiskan® reader (Thermo life science MS 200-240).

The calculation of the % of cell viability is as follow:

$$\% \text{ of cell viability} = \frac{(A_{test\ \lambda 570\ nm})}{(A_{control\ positive\ for\ growth\ \lambda 570\ nm})} \times 100$$

2.4. Microarray experiments

2.4.1. Cell treatment

Three different batches of HUVEC cells (biological triplicate) and two different batches of NL20 cells (biological duplicate) were used for the following treatment and each batch was investigated only one time. See Table 2.

For each cell batch (HUVEC and NL20) three conditions were investigated:

1. Cells at $5x10^5$ cells/ml were treated with medium alone
2. Cells at $5x10^5$ cells/ml were treated with PT (batch 2123) (2.5 µg/ml)
3. Cells at $5x10^5$ cells/ml were treated with detoxified PT (batch 2120) (2.5 µg/ml)

All the cells were seeded and treated in T175 cell culture flasks and were incubated for 6 h at 37^0C in a humidified atmosphere that contains 5% CO_2. Treated cells were harvested and rinsed twice in PBS. See Table 2.

Table 2. Design of microarray experiments

Cell batches →	Cell treatments →	RNA extraction samples →	Arrays hybridised with radioactive cDNA →	Gene lists (Genes differentially expressed by over 2 fold relative to the same gene in control group)
HUVEC cells batch 1	Cells + medium	1.RNA Control 1	Array Control 1	
	Cells + PT	2.RNA PT 1	Array PT 1	1.Genes up-regulated by PT1 2.Genes down-regulated by PT1
	Cells + dPT	3.RNA dPT 1	Array dPT 1	3.Genes up-regulated by dPT1 4.Genes down-regulated by dPT1
HUVEC cells batch 2	Cells + medium	4.RNA Control 2	Array Control 2	
	Cells + PT	5.RNA PT 2	Array PT 2	5.Genes up-regulated by PT2 6.Genes down-regulated by PT2
	Cells + dPT	6.RNA dPT 2	Array dPT 2	7.Genes up-regulated by dPT2 8.Genes down-regulated by dPT2
HUVEC cells batch 3	Cells + medium	7.RNA Control 3	Array Control 3	
	Cells + PT	8.RNA PT 3	Array PT 3	9.Genes up-regulated by PT3 10.Genes down-regulated by PT3
	Cells + dPT	9.RNA dPT 3	Array dPT3	11.Genes up-regulated by dPT3 12.Genes down-regulated by dPT3
NL20 cells batch 1	Cells + medium	1.RNA Control 1	Array Control 1	
	Cells + PT	2.RNA PT 1	Array PT 1	1.Genes up-regulated by PT1 2.Genes down-regulated by PT1
	Cells + dPT	3.RNA dPT 1	Array dPT 1	3.Genes up-regulated by dPT1 4.Genes down-regulated by dPT1
NL20 cells batch 2	Cells + medium	4.RNA Control 2	Array Control 2	
	Cells + PT	5.RNA PT 2	Array PT 2	5.Genes up-regulated by PT2 6.Genes down-regulated by PT2
	Cells + dPT	6.RNA dPT 2	Array dPT 2	7.Genes up-regulated by dPT2 8.Genes down-regulated by dPT2

2.4.2. Total RNA extraction

Total RNA extraction was carried out on the treated cells using RNeasy maxi kit (Qiagen 75162) according to manufacturer's instructions. Extracted RNA was then treated with 630 Units of DNase I (114 U/µl Invitrogen 18047-019) for 30 min at 37^0C. The total RNA was further precipitated for 2 h in the presence of 0.5 M ammonium acetate and 2.5 volumes of ethanol 100% (v/v) at -80^0C. The RNA pellet after centrifugation was washed twice in 70% ethanol and the pellet resuspended in RNase free water (Promega). Optical density was measured for each sample at 260 nm to determine the quantity of RNA. Taking the correlation 1 A_{260} unit of ssRNA = 40 µg/ml H_2O, the concentration of RNA sample were calculated. Optical density was also measured at 280 nm to allow the calculation of the ratio A_{260}/A_{280}. Samples with a ratio < 1.8 were discarded. According to the design of cell treatments (see table 2) a total of nine different RNA samples were extracted from each HUVEC cell culture: three different batches of cells treated with medium alone, PT at 2.5 µg/ml, and dPT at 2.5 µg/ml. A total of six RNA samples were extracted from NL20 cells: two different batches of cells treated with medium alone, PT at 2.5 µg/ml, and dPT at 2.5 µg/ml.

2.4.3. Microarray template

Plastic human 12K microarray (BD biosciences BD Atlas^{tm} 634811) was used for the experiments. This plastic membrane was coated with oligonucleotides representing 12,000 human genes. Each oligonucleotide was present in duplicate on the plastic membrane. Microarray experiments were performed according to the manufacturer's instructions and are briefly described as follows. All the reagents were obtained from BD biosciences unless otherwise stated.

2.4.4. Preparation of cDNA

2.4.4.1. Annealing primers

Total RNA 50μg / treatment (a total of 9 for HUVEC cells and 6 for NL20 cells) was incubated with 1 μl of random primers, 4 μl of Oligo(dT) primers (Promega, 500 μg/ml) in a total volume of 16 μl for 10 min at 65^{0}C followed by 10 min at room temperature, according to the manufacturer's protocols (BD biosciences BD Atlas^{tm} plastic human 12K microarray 634811).

2.4.4.2 Reverse transcription

To a 16 μl of annealing mixture from 2.4.4.1, 8 μl of 5x BD powerscript reaction buffer, 3 μl dNTP (dATP, dCTP, dTTP and dGTP at 10 mM, Promega), 1 μl of 10x DTT, 10 μl (50μCi) of radioactive ^{33}PdCTP (Amersham BF1005) and 2 μl of reverse transcriptase enzyme were added. Reaction mixtures were incubated for 1 h at 42^{0}C to synthesise the cDNAs. After this, 4 μl of 10x terminal reaction solution was added. Populations of radioactive cDNA were purified by column chromatography, according to the manufacturer's protocols (BD biosciences BD Atlas^{tm} plastic human 12K microarray 634811). Populations of radioactive cDNA were synthesised from RNA extracted from cells treated according to the design described in table 2. Therefore, a total of 15 populations of radioactive cDNA were synthesised. Nine populations of radioactive cDNA were related to the three different batches of HUVEC cells treated with medium alone, PT at 2.5 μg/ml, and dPT at 2.5 μg/ml. Six populations of radioactive cDNA we are related to the two different batches of NL20 cells treated with medium alone, PT at 2.5 μg/ml, and dPT at 2.5 μg/ml.

2.4.4.3 Hybridization

Each population of radioactive cDNA was hybridised to an individual plastic membrane (BD bioscience 634811). This plastic membrane was coated with

oligonucleotides representing 12,000 human genes. Each oligonucleotide was present in duplicate on the plastic membrane. According to the manufacturer's protocol, radioactive cDNAs were hybridised to one plastic membrane at 60^0C overnight in the hybridisation buffer (BD bioscience).The hybridised arrays were exposed to a phosphorous screen for two weeks at room temperature. The radioactive hybridised signals were detected using a Typhoon scanner (Typhoon 9410, Molecular dynamics). The Typhoon scanner takes a picture of the hybridised plastic membrane where one spot corresponds to the expression of one gene. On this picture each dark spot corresponds to a radioactive cDNA hybridised to an oligonucleotide probe coated on the membrane. Three hybridisation experiments were carried out for HUVEC study (related to the three batches studied) and two hybridisation experiments were carried out for NL20 study (related to the two batches studied). In each experiment, three populations of radioactive cDNA (related to treatment design: medium alone, PT 2.5 µg/ml and dPT 2.5 µg/ml) were hybridised to three different plastic membranes.

2.4.5. Microarray analysis

2.4.5.1. Template alignment

Using Atlasimage® software (version 2.7, Clontech lab), the image of hybridised arrays on each membrane showing the radioactive signals was collected and aligned to gene templates. For each array, the signals with discordant or absent results were discarded and radioactive intensities of spots were quantified. Therefore the software allowed us to make a relation between a spot and the name of the gene investigated (according to the template) and also allowed us to have the intensities of a spot converted in numbers called raw data. Because each array had two spots for one gene, each time an array was hybridised, two sets of data for each gene were

obtained. The resulting data were then normalized, filtered and analysed using GeneSpring® software (version 7, Silicongenetics).

2.4.5.2. Normalisation and calculation of statistical significance of internal control.

GeneSpring® software was used to analyse the raw data, including normalisation of signals using a global normalisation method to subtract the background and the statistical significance of the internal replicates. Two-colour normalisations were carried out. The measured intensity for each gene was divided by its control channel value in sample(s) when less than 100 genes showed signals per region; if the control channel value was below 5 then 5 was used instead. If the control channel and the signal channel values were both below 5 then no data were reported. When more than 100 genes showed signals per region, a Lowess curve was fitted to the log-intensity versus log-ratio plot. 20.0% of the data was used to calculate the Lowess fit at each point. This curve was used to adjust the control value for each measurement. If the control channel value was lower than 10 then 10 was used instead. After normalisation, the intensities of the two spots for each gene were averaged to give the intensity of a gene. Robustness of the intensity of a gene was calculated using a statistical programme (Cross-gene error model) built in to the GeneSpring software.

2.4.5.3. Filtering of the data

After normalisation of the results, the data generated were displayed in scatter plots. The scatter plot displayed each gene as a dot and placed it in the graph according to the signals recorded on the control membrane (X-axis) and the treatment membrane (Y-axis). Therefore, the ratio of the expression level was clearly illustrated and up-regulated and down-regulated genes were represented according to a colour code

explained in the Results chapter (e.g. Fig. 7 and 8). At this stage, genes were selected, on the basis of expression level, with the aid of filters available in the software. Gene filtered must be in agreement with several conditions: 1) The intensity of the gene averaged from the two spots (internal control) must be considered robust according to the programme, 2) The intensity of the gene must increase or decrease, after PT or dPT treatment, by over two-fold relative to the same gene in the control group. According to the experiment design shown in table 2, filtered genes were classified in gene lists. A total of 12 gene lists were produced for the genes up and down-regulated in HUVEC cells treated with PT or dPT in microarray experiment numbers 1, 2 and 3 (triplicate). A total of 8 gene lists were produced for the genes up and down-regulated in NL20 cells treated with PT and dPT in microarray experiment numbers 1 and 2 (duplicate).

2.4.5.4. Reproducibility between microarray experiments.

The Venn diagram option of GeneSpring software allowed analysis of the content of the gene lists produced on the scatter plots. The Venn diagram allows the comparison of three different gene lists simultaneously. Therefore, genes present in more than one list or in one list only can be highlighted. The first part of the analysis was based on the number of genes differentially expressed by different treatments across the triplicate or replicate experiments for HUVEC or NL20 cells, respectively. To investigate the reproducibility between microarray experiments, the percentage of similitude across was calculated as follows:

$$\% \text{ of similitude} = \frac{\text{Genes up- or down-regulated by PT or dPT in experiment 1, 2 or 3} \times 100}{\text{Total of genes up- or down-regulated by PT or dPT in experiment 1, 2 or 3}}$$

2.4.5.5. Selection of candidate genes

The second part of the analysis was to select the genes differentially expressed by PT and by dPT. These genes were selected using the Venn diagram of the HUVEC results as those up-regulated by PT treatment but not by dPT treatment and present in at least two experiments. Ultimately, gene candidates were selected for confirmation by RT-PCR.

2.5. Semi-quantitative RT-PCR

2.5.1. Cell treatments

2.5.1.1. PT and detoxified PT treatments on HUVEC and NL20 cells.

The same batches of cells as used in the microarray experiments were treated and then RNA was extracted in order to perform microarray and semi-quantitative RT-PCR experiments, as described in section 2.4.1.

2.5.1.2. Pertussis toxin and interleukin-1 beta (IL-1 beta) on HUVEC cells.

In an attempt to get closer to *in vivo* conditions and to increase the sensitivity of signal detection, PT treatments in association with IL-1 beta were carried out on HUVEC cells. Cells at $5x10^5$ cells/ml were incubated in the presence or absence of PT (100 ng/ml) at 37^0C in a humidified atmosphere containing 5% CO_2. After incubation for 18h, the cells were stimulated for 4 h with IL-1β (5 IU/ml) (Sadeghi *et al.*, 2000). Four different groups of cells were treated:

Group 1: for 22 h with medium alone

Group 2: for 18 h with medium alone + for 4 h with IL-1β (5 IU/ml)

Group 3: for 18 h with PT (100 ng/ml) + for 4 h with medium alone

Group 4: for 18 h with PT (100 ng/ml) + for 4 h with IL-1β (5 IU/ml).

2.5.1.3. Pertussis toxin and tumor necrosis factor alpha (TNF alpha) on HUVEC cells.

Cells at $5x10^5$ cells/ml were incubated in the presence or absence of PT (100 ng/ml) at 37^0C in a humidified atmosphere containing 5% CO_2. After incubation for 18 h, the cells were stimulated for 6 h with TNFα (20 IU/ml)(Bernot *et al.*, 2005). Four different groups of cells were treated:

Group 1: for 24h with medium alone

Group 2: for 18h with medium alone + for 6 h with TNFα (20 IU/ml)

Group 3: for 18h with PT (100 ng/ml) + for 6 h with medium alone

Group 4: for 18h with PT (100ng/ml) + for 6h with TNFα (20 IU/ml).

2.5.2. Total RNA extraction

Extraction of total RNA was carried out on the treated cells as in section 2.4.2.

2.5.3. Semi-quantitative RT-PCR

Total RNA digested with DNAse I (114 U/µl, Invitrogen 18047-019), was diluted in RNAse-free water to 2 µg/gene investigated, 1 µg/gene investigated, 0.1µg/gene investigated and 0.01 µg/gene investigated. Diluted RNA was then reverse-transcribed for 90 min at 42^0C with 2 µl Oligo(dT) primers at 0.1 µg/µl (Promega) and 1 µl (200U) of Superscript II RT (Invitrogen 18064-014), 4 µl RT buffer (x5), 1 µl dNTPs (Promega, 10 mM) and 2 µl DTT 0.1M (Invitrogen). The resultant cDNA (10 µl/gene investigated) was used for PCR using a heating step of 94^0C for 2 min and 30 cycles of 1 min at 96^0C, 1 min at 52^0C and 1 min at 72^0C and a final step at 72^0C for 7 min. The PCR mixture for each gene investigated was 2µl of each primer (10 pMol/µl), 5 µl of enzyme buffer (x10), 1 µl $MgCl_2$ (25 mM), 5 U Taq polymerase (Promega M1665). PCR amplification products were electrophoresed in agarose gels

(1% (w/v) in TBE buffer (x1): Tris 109 g/ml, boric acid 55.6 g/ml, EDTA 9.3 g/ml pH 8.3) containing ethidium bromide (5% (v/v)).

The mRNA sequences of the genes selected for RT-PCR from the microarray analysis were assembled from the NCBI database (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov). Primers (Invitrogen) were designed based on the above sequences using Gene runner 3.05® software (See Table 3). Primers for the genes VCAM-1 (vascular cell adhesion molecule-1), ICAM-1 (intercellular adhesion molecule-1) and E-selectin (endothelial adhesion molecule 1) were identical to the sequences used by Meagher *et al.* (1994) (Table 3).

Table 3. Genes targetted by semi-quantitative RT-PCR.

Genbank (accession number)	Gene name	Annealing temperature (^{0}C)	Expected fragment product (bp)	Primer sequences
L05515	cAMP response element-binding protein mRNA (CRE-Bp1)	56	567	5'-CATCACCACTCCCATTCC-3' and 5'-TCTGTGAGTGGTGAGCTG-3'
NM_005567	lectin, galactoside-binding, soluble, 3 binding protein (Galectin 3)	54	556	5'-CCTGTTCCAGAAGAAGACT-3' and 5'-TCTTCGAGCTGTTGGTGTC-3'
NM_016103	GTP-binding protein Sara (SAR1)	56	552	5'-ACAGTGGTTTCAGCAGTGT-3' and 5'-ATCCAGCGGAAGCCTTCT-3'
NM_000742	Cholinergic receptor, nicotinic, alpha polypeptide 2 (neuronal) (CHRNA)	56	537	5'-TGCTGCTCATCACTGAGAT-3' and 5'-TTCACCGAAGAGTCAGCAT-3'
NM_001383	diphthamide biosynthesis protein 2, S. c, homolog-like 1 (DPH2L1)	56	549	5'-CGGATAGACACTACACAC-3' and 5'-ATCCACCTCAGGAAGTAG-3'
NM_002046	glyceraldehyde-3-phosphate dehydrogenase (GapDH)	58	500	5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTG-3'
NM_001078	vascular cell adhesion molecule-1 (VCAM-1)	56	259	5'-ATGACATGCTTGAGCCAGG-3' and 5'-GTGTCTCCTTCTTTGACACT-3'
NM_000201	Intercellular adhesion molecule-1 (ICAM-1)	55	237	5'-TATGGCAACGACTCCTTCT-3' and 5'-CATTCAGCGTCACCTTGG-3'
NM_000450	Endothelial adhesion molecule 1 (E-selectin)	57	254	5'-CTCTGACAGAAGAAGCCAAG-3' and 5'-ACTTGAGTCCACTGAAGCCA-3'

2.6. Cell migration assay

Migration of the endothelial cells (HUVEC) was examined using a 48-well chemotaxis chamber (Neuroprobe AP48) and polyvinylpyrrolidone-free polycarbonate membranes with 8-µm pores (Neuroprobe PFB8-50), coated with 6.5 µg/ml fibronectin (Sigma F1141)(Figure 3).

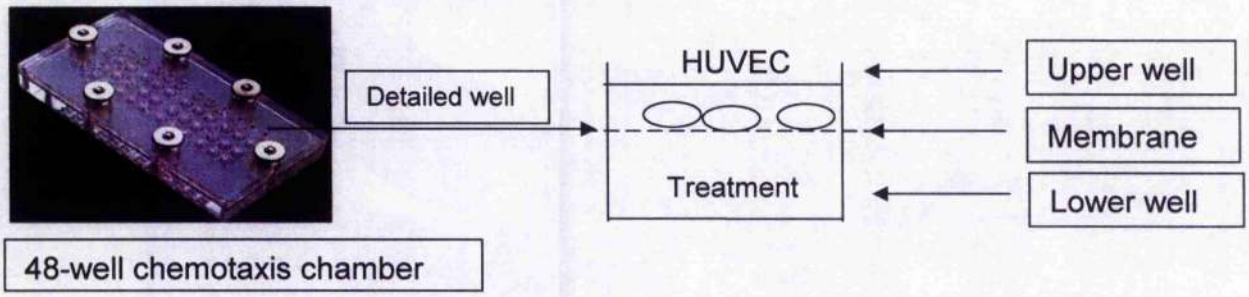


Figure 3. The 48-well chemotaxis chamber.

Pertussis toxin (2133) was diluted in phenol red-free endothelium basal medium (EBM), containing 1% (w/v) bovine serum albumin (BSA) (Cambrex CC-3129) and was then added to the lower chambers at concentrations ranging from 0 to 10 µg/ml. EBM with 1% (w/v) BSA was used as a negative control and the medium supplemented with 10% (v/v) FCS (Invitrogen 10108-165) or LPS (100 ng/ml) were used as positive controls for the experiment. Cell suspensions (50 µl) ($1x10^6$ cells/ml) in Phenol Red free EBM Medium were added to the upper wells. Four time points (1, 2, 4 and 6 h) were investigated initially but 4 h was chosen because it gave the highest number of migrated cells in the treatment wells. Thus, after incubation for 4 h at 37^0C in a humidified chamber containing 5% CO_2, the membrane was removed and placed in methanol for 30 s to fix the cells. The cells were stained in eosin for 60 s (cytoplasm staining) and then placed in Methyl Thionins (Prodiff, Braidwood laboratories) for a further 60 s (nucleus definition). The membrane was rinsed in PBS and the top side of the membrane was wiped using wet tissue in order to remove unmigrated cells. The number of cells migrated into and through the membrane was

counted for, at magnification x 40, in 3 microscopic fields per well under a microscope (Olympus XS51).

3. RESULTS

3.1. Effects of pertussis toxins on mammalian cells

The aim of the cytotoxic assays was to determine the concentration of toxins and the time of incubation that would give a maximal biological effect, but also maintain cell viability (in this case not kill more than 20% of the cells) (see section 3.1.2.2). That concentration was then used to perform the microarray experiments.

3.1.1. Effects of toxin preparations on cell morphology.

The morphology of cells was monitored at four time points, 0, 6, 24 and 48 h for each preparation of PT, dPT and LPS. The concentrations investigated ranged from 0, 0.3, 0.6, 1.25, 2.5, 5 to 10 μg/ml. Four PT toxin preparations, which differed in concentration and buffer system, were tested for their suitability for the assay. Their associated buffers were also tested as controls. All the toxins were diluted in the culture medium when required. It was noticed that some buffers used for the toxin preparations were found to induce the formation of cell aggregates. This phenomenon was observed for the buffer used in PT PAC 090, PT 90/518 and PT JNIH-5. The buffer used for preparation of PT PAC2133 did not cause this effect. Therefore, PT PAC2133 was chosen for subsequent studies. The results are presented in Figure 4, which shows the morphologies of four cell lines treated with 2.5 μg/ml of PT. All cell lines were seeded at $5x10^6$ cells/ml and incubated for 6 h at 37^0C in a humidified atmosphere containing 5% of CO_2. Untreated (control, medium alone) Jurkat cells (Fig. 4 A) were evenly distributed in the field (left). Cells treated with PT at 2.5 μg/ml for 6 h were significantly affected, with reduction of cell surface

and the formation of cell aggregates. For NL20 (Fig. 4 B), SHSY5Y (Fig. 4 C) and HUVEC (Fig. 4 D) cells, the untreated cells were generally well spread. Aggregation and reduction in surface area was observed in the cells treated with PT at concentrations ranging from 2.5 μg/ml (Fig. 4) to 10 μg/ml in a dose dependent manner with the highest response obtained at 10 μg/ml (data not shown). Cells treated with dPT and LPS, at these same concentration, had morphologies comparative to the control cells.

3.1.2. Cytotoxic effects

3.1.2.1. Effect of toxins on cell lines assessed by Alamarblue

The cell viability of four cell lines (Jurkat, NL20, SHSY5Y and HUVEC) treated with PT, dPT and LPS was monitored using the Alamarblue technique at four time points 0, 6, 24 and 48 h. The toxin concentrations tested ranged from 0, 0.3, 0.6, 1.25, 2.5, 5 to 10 μg/ml. For each toxin, the percentage of viability calculated at all concentrations tested was similar at each time point and no killing was observed (data not shown). Consequently no dose-dependent effect was observed and it was concluded that the test was not suitable for our study

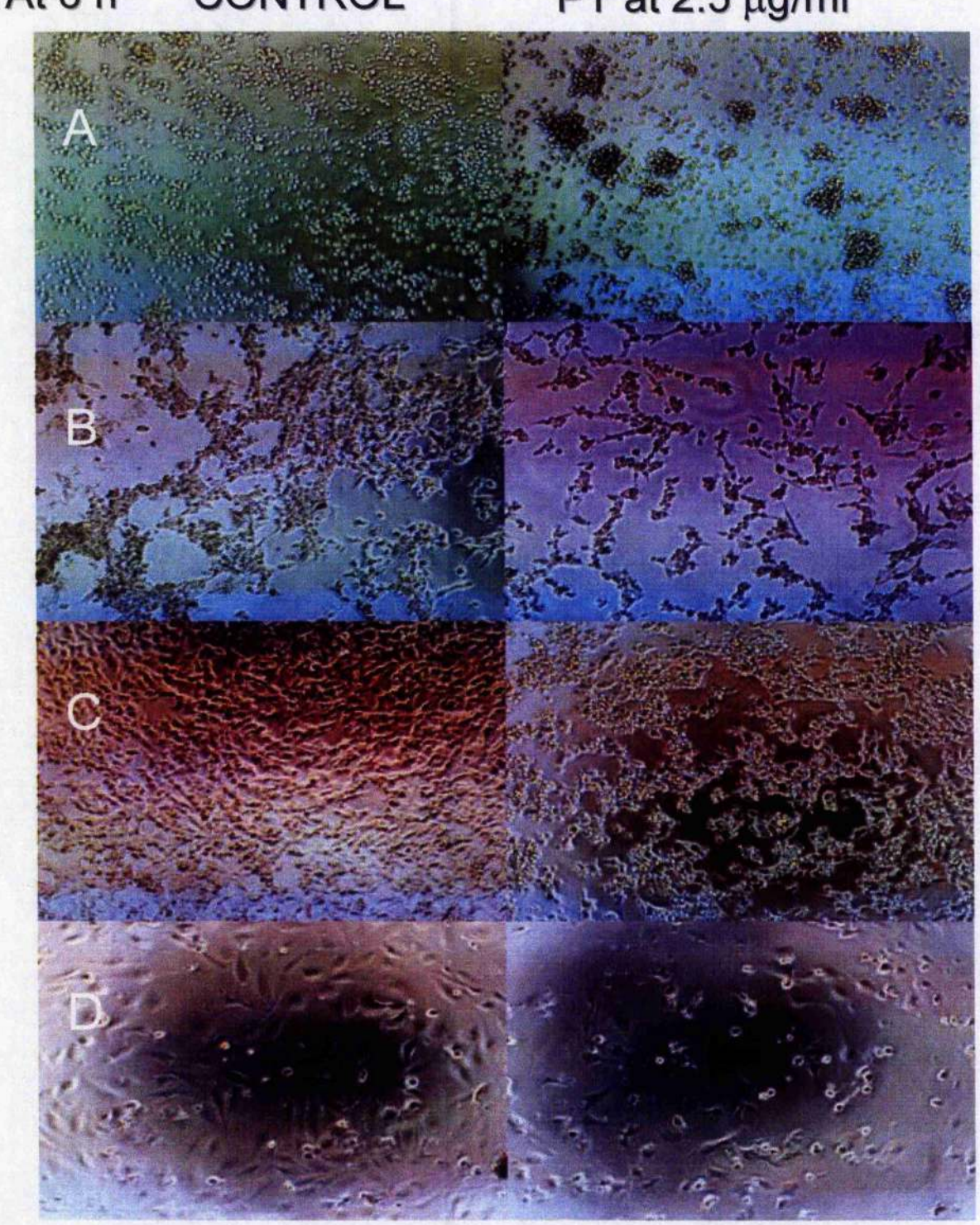


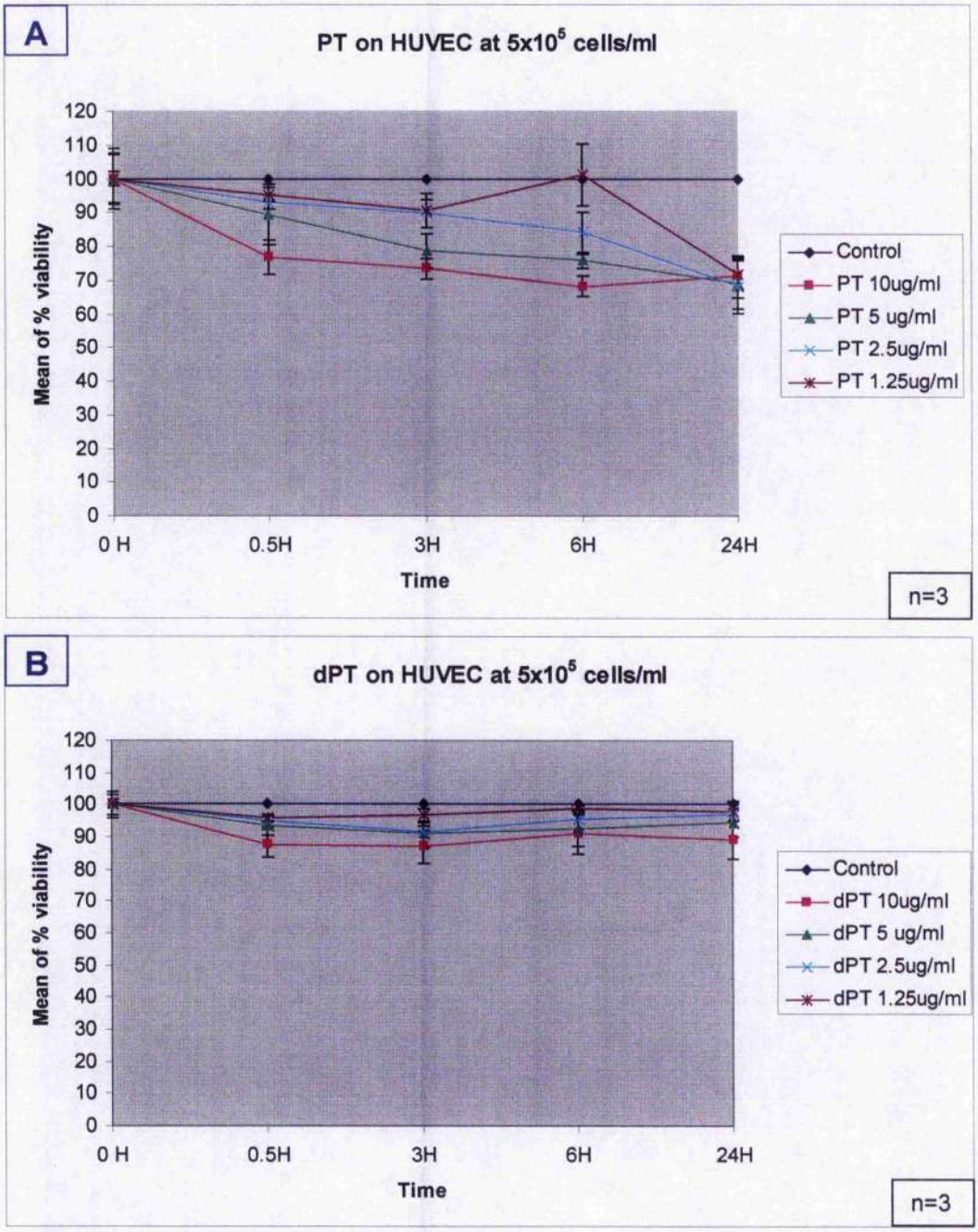
Figure 4. Morphology of different cell line after incubation for 6 h with or without PT at 2.5 µg/ml.

(A) Jurkat cells treated with PT (right) are more aggregated than in the control (left); (B) NL20 cells treated with PT are less spread than in the control; (C) SHSY5Y cells treated with PT are less spread than in the control; (D) HUVEC primary cells treated with PT are more detached and the surface area of the cells is much smaller than in the control.

3.1.2.2. Effect of toxins on cell lines assessed by MTT.

The cell viability of two cell types (HUVEC and NL20) treated with PT and dPT was monitored using the MTT technique at 0, 0.5, 6 and 24 h. The toxin concentrations ranged from 0, 1.25, 2.5, 5 to 10 μg/ml. Figures 5 and 6, for HUVEC and NL20 cells respectively, show the mean percentage of viability calculated from three independent experiments. According to the results illustrated in Fig. 5B and Fig. 6B, dPT does not show any marked killing at any time point and at all the concentrations tested for both HUVEC and NL20 cells. On the other hand, Figs. 5A and 6A show a reduction of the percentage of cell viability when cells were treated with PT. For both cell types, the reduction of cell viability showed a dose-dependent pattern, with a maximal effect reached with 10 μg/ml of PT. This gave a reduction in cell viability of 30% for HUVEC and 35% for NL20. Regarding the time course of the killing effect, killing was observed within 30 min of incubation. However, the maximum killing effect was reached after 6 h for HUVEC cells and 3 h of incubation for NL20 cells.

The aim of the cytotoxicity assay was to determine a suitable toxin concentration for use in the microarray experiments. Cells showing modifications caused by PT treatment (i.e. morphological changes, cell death) were of interest, but a large number of transcriptionally-active cells and consequently not dead cells were required. Thus a toxin concentration of 2.5 μg/ml was chosen. This would induce a reduction of the cell viability of about 15% for HUVEC and 20% for NL20 after incubation for 6 h.

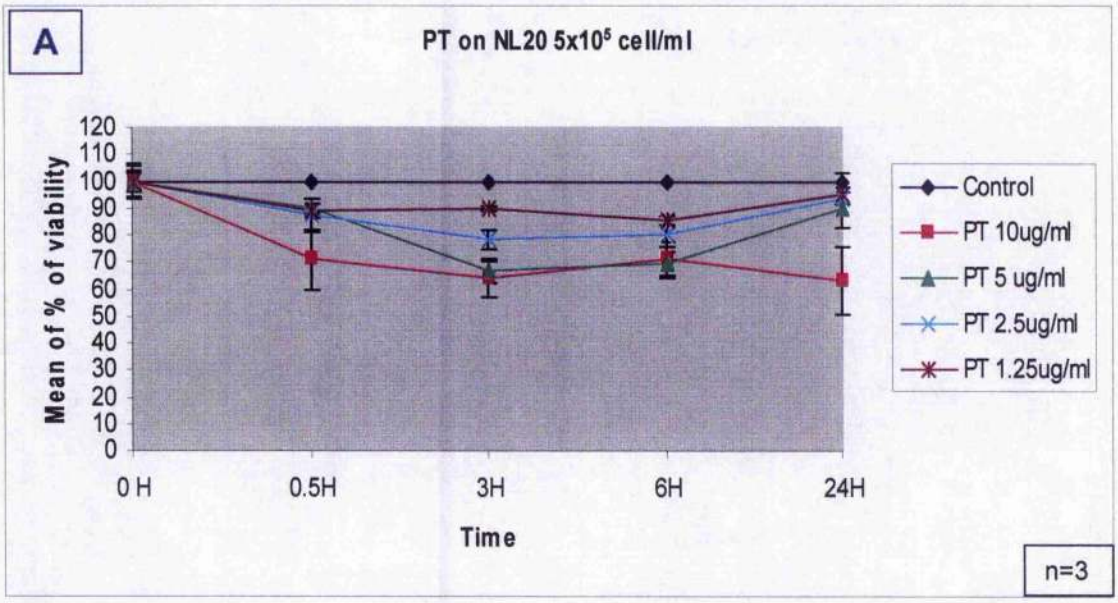


PT

dPT

Figure 5. Cytotoxicity test: Cell titer96® non-radioactive cell-proliferation assay on HUVEC cells treated with pertussis toxin (A) or detoxified pertussis toxin (B). Data are the mean % viability calculated from three independent experiments and bars represent the standard deviations.

PT



dPT

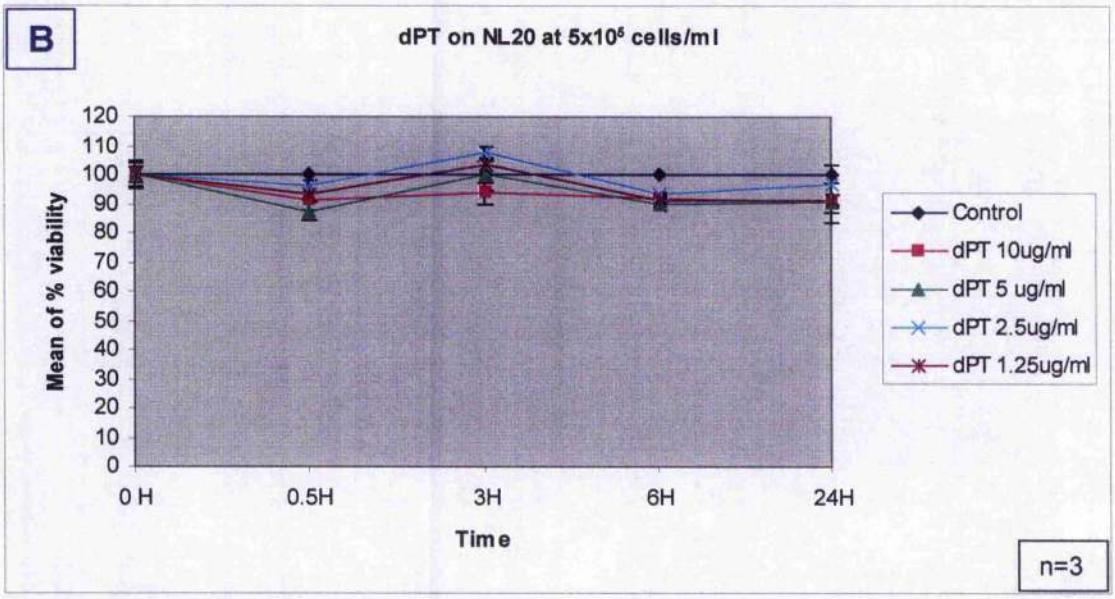


Figure 6. Cytotoxicity test: Cell titer96® non-radioactive cell-proliferation assay on NL20 cells treated with pertussis toxin (A) or detoxified pertussis toxin (B). Data are the mean % viability calculated from three independent experiments and bars represent the standard deviations.

3.2. Microarray analysis

HUVEC and NL20 cells, at $5x10^5$ cells/ml, were treated for 6 h with medium alone (control), PT or dPT at a concentration of 2.5 µg/ml. Total RNA was extracted from the cells and microarray analysis was performed. The data produced were normalised and further analysed. The aim of this project was to identify any potential gene markers of toxicity. Therefore, after analysis of the reproducibility of the technique, gene lists that were generated for the two toxin preparations were compared, to determine genes which were up-regulated after PT treatment only.

3.2.1. Gene list production and experimental consistency

Figs 7 and 8 show scatter plots representing the gene expression profiles of HUVEC and NL20 cells, generated using GeneSpring software. On the scatter plot, three diagonals are drawn, they symbolize three different sections of the plot, encompassing those genes that were up-regulated by at least two-fold (>2, in red), those that were unchanged (<2 fold change in yellow) and those that were down-regulated by at least two-fold (>0.5 in green). For each experiment (HUVEC: Experiments 1, 2 and 3; NL20: experiments 1 and 2) and each treatment (PT or dPT) lists of these up- or down-regulated genes were produced. Therefore, a total of 12 lists for HUVEC and 8 lists for NL20 cells were generated and the numbers of these genes are shown in Tables 3 and 4.

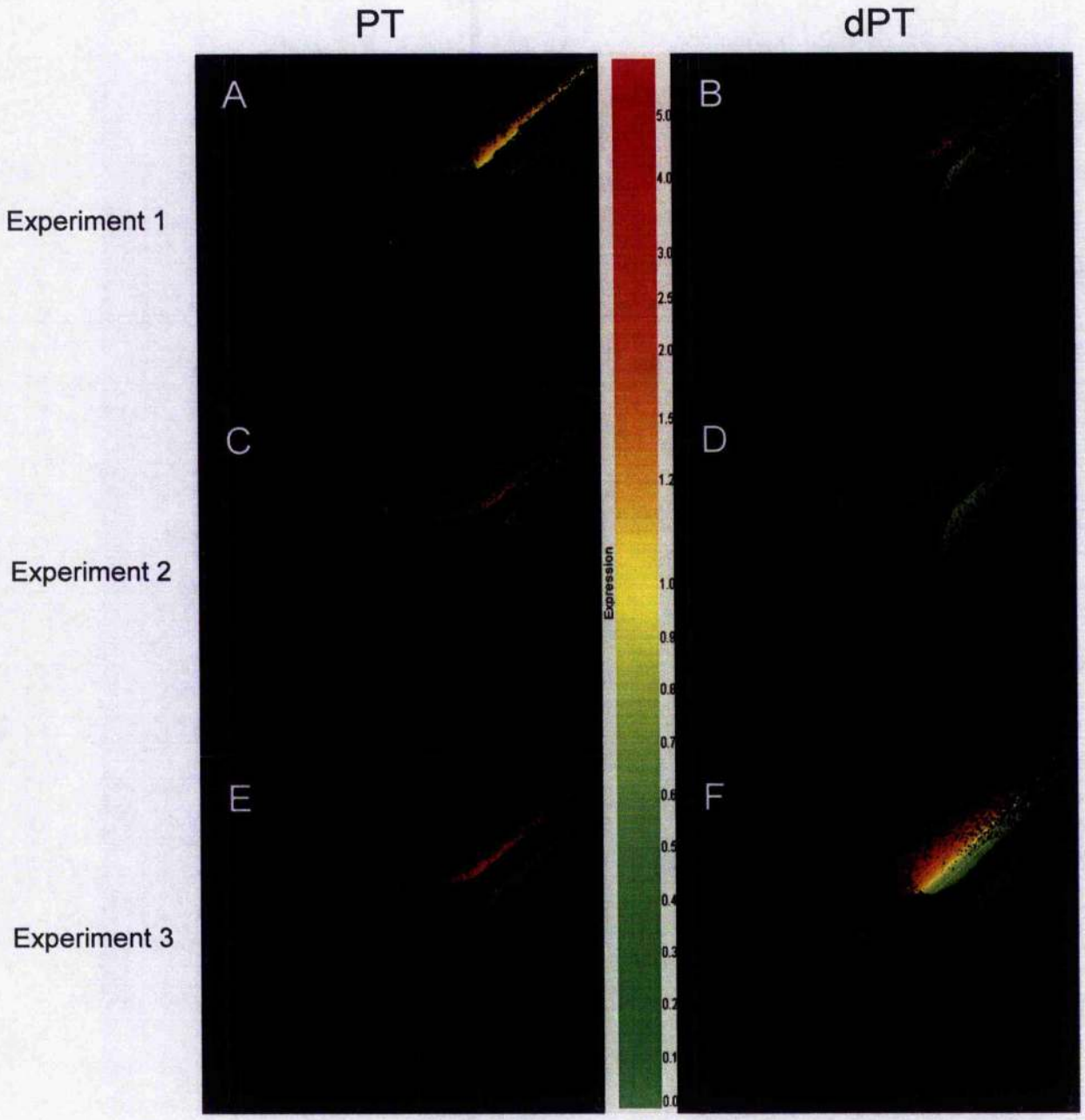


Figure 7. Scatter plots of HUVEC gene expression profiles after PT and dPT treatments

Expression profiles were obtained for each of 3 independent experiments (Experiments 1, 2 and 3) after treatment of HUVEC cells with PT (A, C and E) and dPT (B, D and F) at 2.5 μg/ml for 6 h. On the scatter plot, one dot corresponds to one gene and it is placed on the graph according to the signal recorded on the control membrane (X-axis) and on the treatment membrane (Y-axis). Colour code is a guide to visualise the differential expression level of the genes. Therefore those genes that were >2-fold up-regulated are shown in red, genes unchanged are in yellow and genes that were >0.5-fold down-regulated are in green. From these scatter plots, the genes for each treatment and each experiment with an expression level of 2 or above (Up-regulated genes) and with an expression level of 0.5 or below (Down-regulated genes) were selected.

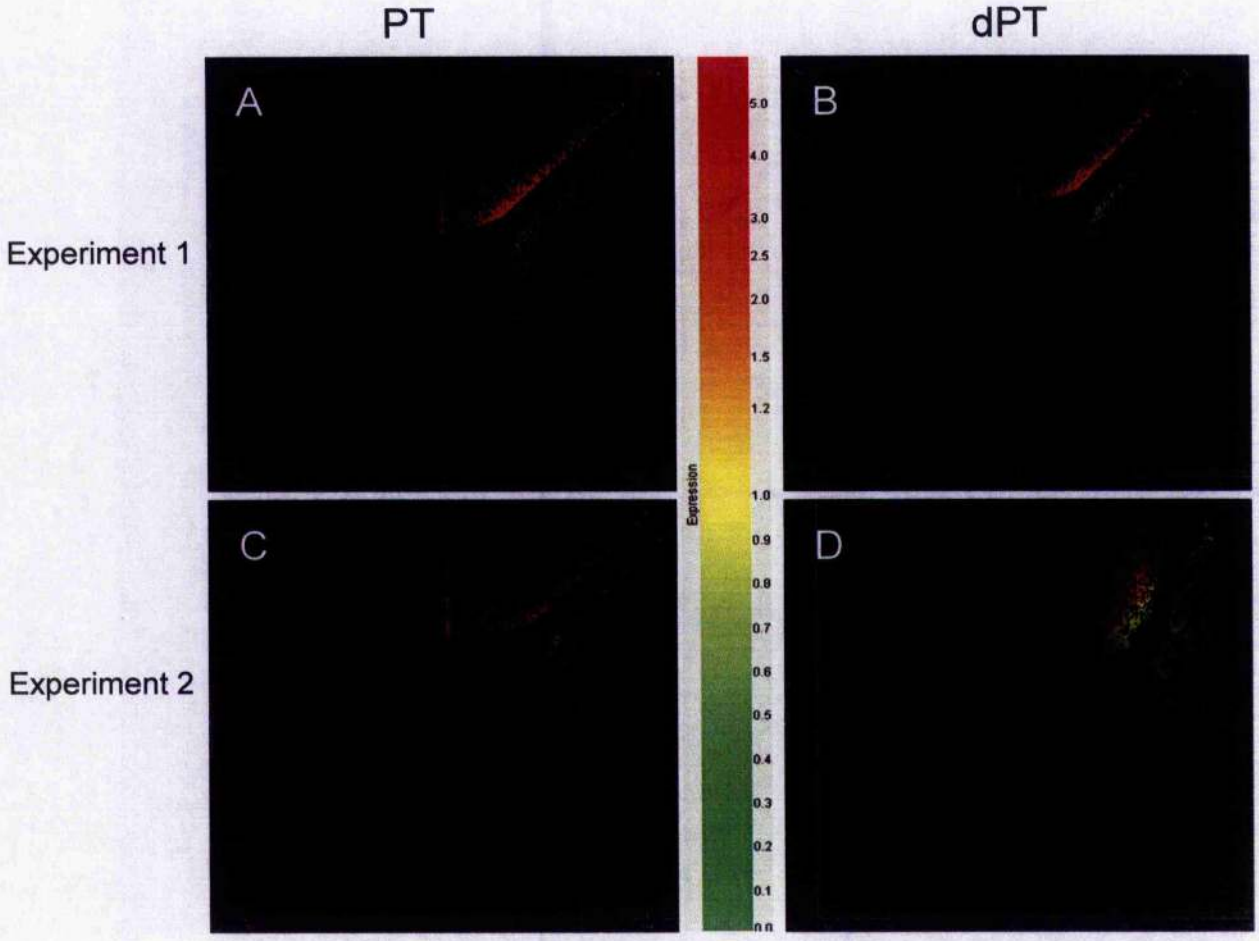


Figure 8. Scatter plots of NL20 gene expression profiles after PT and dPT treatments

Expression profiles were obtained for each of 2 independent experiments (Experiments 1 and 2) after treatment of NL20 cells with PT (A and C) and dPT (B and D) at 2.5 μg/ml for 6 h. On the scatter plot, one dot corresponds to one gene and it is placed on the graph according to the signal recorded on the control membrane (X-axis) and on the treatment membrane (Y-axis). Colour codes are as described in the legend in Figure 7.

Table 4. Gene lists related to HUVEC microarrays

HUVEC genes	PT (Experiment 1)	PT (Experiment 2)	PT (Experiment 3)	dPT (Experiment 1)	dPT (Experiment 2)	dPT (Experiment 3)
Up-regulated	48	179	349	125	53	811
Down-regulated	77	59	125	215	400	797

Table 5. Gene lists related to NL20 microarrays

NL20 genes	PT (Experiment 1)	PT (Experiment 2)	dPT (Experiment 1)	dPT (Experiment 2)
Up-regulated	471	144	330	548
Down-regulated	114	98	103	343

According to the results presented in Tables 3 and 4, the number of differentially expressed genes across different experiments was variable. For instance, the number of up-regulated genes among the PT HUVEC microarrays was approximately 7-fold higher in experiment 3 (349 genes) compared to experiment 1 (48 genes). The range of such variation reached a higher level (~15 times) among microarrays after dPT treatment: 53 in experiment 2 and 811 in experiment 3. On the other hand, the variation is more modest among the down-regulated genes in the HUVEC microarrays, 1.6 and 3.7 times for PT and dPT, respectively.

In the experiments with NL20 cells, the variation of gene numbers differentially expressed between replicates was low: 3.2 times for genes up-regulated by PT, 1.6

times for genes up-regulated by dPT, 1.16 times for genes down-regulated by PT and 3.3 times for genes down-regulated by dPT.

3.2.2. Reproducibility

The 12 gene lists produced from the HUVEC microarray experiments and the 8 gene lists produced from the NL20 microarray experiments were further analysed using Venn diagrams (Figs 9,10), an option available on GeneSpring software. In the Venn diagram, genes are coloured according to their membership in one or more gene lists. By comparing the three gene lists related to up-regulation after PT treatment of HUVEC cells (Fig. 9), it can be seen, in the white section, that only two of a total of 541 up-regulated genes were identified in all three experiments. Taking the total number (541) of genes up-regulated as 100%, the percentage of similarity between the three experiments can be calculated as 0.37%. No genes were up-regulated in all of the three independent experiments after dPT treatment of HUVEC cells. When the lists of down-regulated genes were compared, in Figs 9 C and D, one gene was identified as common to all three experiments for PT treatment and 16 genes for dPT treatment. Taking the total number (233 for PT treatment and 1234 for dPT treatment) of genes down-regulated as 100%, the percentages of similarity between the three experiments were calculated as 0.43% and 1.29% for PT and dPT, respectively. Thus, none of the triplicates assays identified more than 1.3% of genes in common.

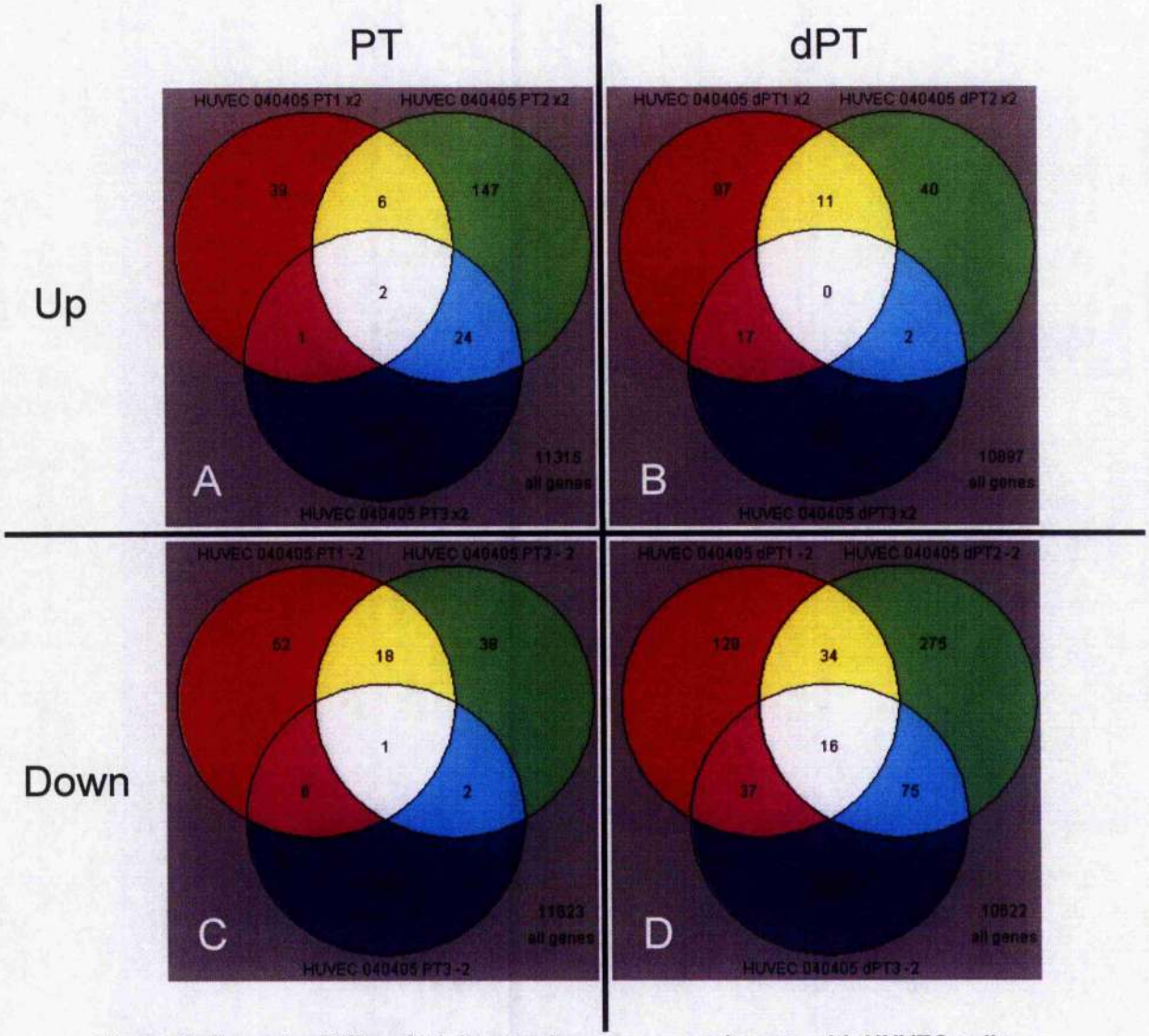


Figure 9. Reproducibility of triplicate microarray experiments with HUVEC cells.

The figure shows the genes from HUVEC cells that were differentially expressed after treatment with PT (A and C) and dPT (B and D) at 2.5 μg/ml for 6h. Up-regulated genes are analysed in diagrams A and B and down-regulated genes in C and D. Each Venn diagram illustrates selected genes from the scatter plots (Fig 7) for experiment 1 (left circle), experiment 2 (right circle) and experiment 3 (bottom circle). The white section represents the number of genes differentially expressed and identified in all 3 experiments. The yellow section represents the number of genes differentially expressed and present in 2 experiments: experiments 1 and 2. The light blue section represents the number of genes differentially expressed and present in 2 experiments: experiments 2 and 3. The pink section represents the number of genes differentially expressed and present in 2 experiments: experiments 1 and 3. The others sections correspond to the number of genes differentially expressed and present in one experiment only: red (experiment 1), green (experiment 2) and blue (experiment 3).

Fig 10 shows the Venn diagrams analysing the gene lists obtained from the replicate NL20 microarray experiments. The white section of (Fig.10, A) shows that 7 genes were commonly up-regulated by PT treatment. A total of 608 genes were up-regulated by PT, so the percentage of similarity was 1.15%. After dPT treatment (Fig.10, B), 41 genes were commonly up-regulated by the detoxified toxin. The total number of genes downregulated is 837, thus the percentage of similarity was 4.9%. The Fig. 10, C and D show the genes down-regulated by PT (C) and dPT (D). For PT treatment, only one gene was found in both experiments and, taking 211 as total number of genes down-regulated, the percentage of similarity was 0.47%. For dPT treatment, 8 genes from a total of 438 were commonly down-regulated and the percentage of similarity was 1.8%. Thus none of the replicates analysed had more than 5% of genes in common.

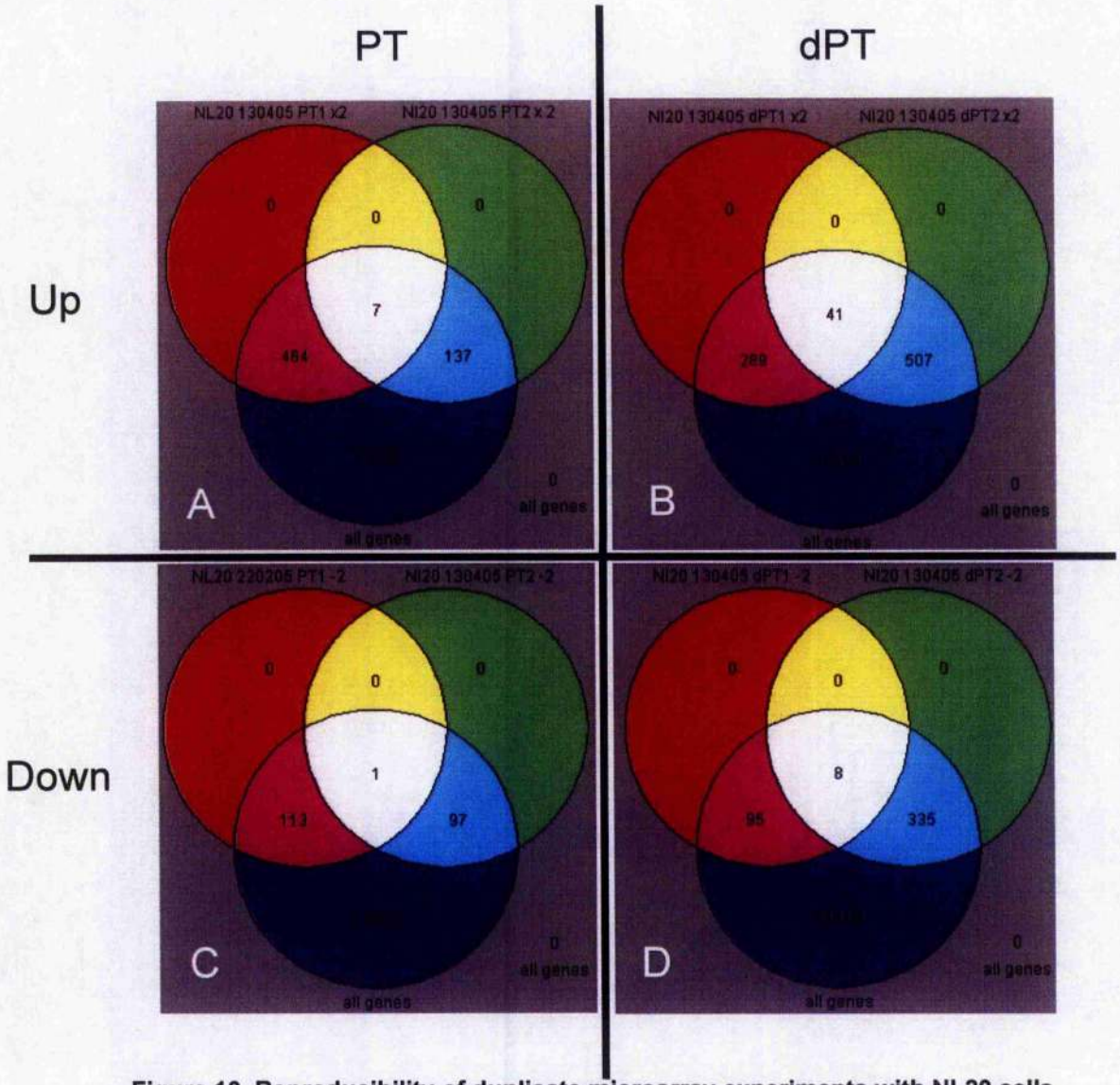


Figure 10. Reproducibility of duplicate microarray experiments with NL20 cells.

The figure shows the genes from NL20 cells that were differentially expressed after treatment with PT (A and C) and dPT (B and D) at 2.5 μg/ml for 6 h. Up-regulated genes are shown in diagrams A and B and down-regulated genes in C and D. Each Venn diagram illustrates selected genes from the scatter plots (Fig 8) for experiment 1 (left circle) and experiment 2 (right circle). The bottom circle shows genes investigated in each experiment (Total 11856 genes). The others sections correspond to the number of genes differentially expressed and present in one experiment only: Pink (experiment 1) and light blue (experiment 2).

3.2.3. Genes of interest

This project was done in an attempt to find a gene marker of toxicity related to pertussis toxin treatment of different human cell lines. So far, the gene list contents have been analysed as numbers but, for those genes commonly up-regulated or down-regulated by PT only, and not by detoxified PT, an analysis of gene function was undertaken. From each gene list related to differential gene expression after PT treatment, the genes also differentially expressed after treatment with chemically detoxified PT in the correspondent microarray experiment were subtracted. The resulting gene lists therefore present genes up-regulated or down-regulated by PT only.

3.2.3.1. Effect of PT on gene expression of HUVEC cells.

3.2.3.1.1. Up-regulation

EXPERIMENT 1/ EXPERIMENT 2/ EXPERIMENT 3

Fig. 11 presents in a Venn diagram the genes upregulated by PT treatment only. The white section of the Venn diagram corresponds to the gene upregulated by PT only and found in all three independent experiments. Only one gene passed the filter and this gene is described as encoding the **cholinergic receptor, nicotinic, alpha polypeptide 2 (neuronal)** (Genbank access NM_000742). cDNA clones encoding the human neuronal nicotinic acetylcholine receptor alpha 2 were originally isolated from thalamus. The deduced 503-amino acid protein contains a signal peptide, 3 N-glycosylation sites and 4 transmembrane regions (Elliott *et al.*, 1996). This gene has been mapped on human chromosome 8 by genomic Southern analysis of hamster/human somatic cell hybrid DNAs (Anand & Lindstrom, 1992). This gene could be an interesting candidate gene for further study because of its relation to the nervous system.

EXPERIMENT 1/ EXPERIMENT 2

In Fig. 11, three genes up-regulated after PT treatment only and found in experiments 1 and 2 are located in the yellow section. Those three genes are:

Lectin, galactoside-binding, soluble, 3 binding protein (NM_005567). The gene encodes a 90K serum protein found in a patient suffering from cancer (Iacobelli *et al.*, 1993). A member of the macrophage scavenger receptor (MSR1) superfamily, the protein binds specifically to the human macrophage-associated lectin (LGALS3) (Koths *et al.*, 1993). In addition, the protein stimulates host defence systems, such as natural killer cell and lymphokine-associated killer cell activities and can induce the secretion of interleukin-2 (Ullrich *et al.*, 1994). This gene may of an interest because of its involvement with the immune system.

GTP-binding protein Sara (NM_016103). The protein encoded by this gene belongs to the Sar1-ADP-ribosylation factor family of small GTPases, which govern the intracellular trafficking of proteins in coat protein (COP)-coated vesicles (Schekman & Orci, 1996). Mutation of this gene has been associated with severe inherited fat malabsorption disorders like chylomicron retention disease, Anderson disease or Marinesco-Sjogren syndrome (Jones *et al.*, 2003). This gene may be of interest because of its similar enzyme activity (ADP-ribosylation) to pertussis toxin and its tissue location (small intestine, liver, muscle and brain).

Diphthamide biosynthesis protein 2, S. cerevisiae, homolog-like 1 (NM_001383). The deletion of this gene is a marker of ovarian epithelial malignancies. The predicted protein was designated DPH2-like because it shares 20% amino acid identity with the *S. cerevisiae* diphthamide biosynthesis-2 protein. The exact function of the protein is unknown (Phillips *et al.*, 1996).

Expression of the above genes was further analysed by RT-PCR (See section 3.3).

EXPERIMENT 1/ EXPERIMENT 3

One gene up-regulated after PT treatment only is present in both experiments 1 and 3. The gene **chromosome 20 open reading frame 81 (NM_022760)** has no known function.

EXPERIMENT 2/ EXPERIMENT 3

By comparing the list of genes up-regulated after PT treatment only, generated from experiments 2 and 3, 15 genes were identified in both experiments.

Cyclin-dependent kinase 8 (NM_001260). The protein encoded by this gene is a member of the cyclin-dependent protein kinase (CDK) family that has been shown to regulate transcription by targeting the CD7/cyclin H subunits of the general transcription initiation factor IIH (TFIIH)(Akoulitchev *et al.*, 2000).

H2B histone family member B (NM_021063) and member E (NM_003521). Histones are basic nuclear proteins that are responsible for the nucleosome structure within the chromosomal fibre in eukaryotes. Studies identified genes encoding member of the H2B class of histones and designated H2Bb and H2BbE (Albig & Doenecke, 1997).

Tubulin, beta, 4 (NM_006086). Microtubules are constituent parts of a diverse variety of eukaryotic cell structures, e.g., the mitotic apparatus, cilia, flagella, and elements of the cytoskeleton. The mouse homologue of human beta-4, M-beta-6 is expressed primarily in neural tissue (Albig & Doenecke, 1997;Burgoyne *et al.*, 1988). In addition, the protein transfected into CHO cells has been reported to decrease the microtubule assembly in a dose-dependent manner (Hari *et al.*, 2003).

Glial fibrillary acidic protein (NM_002055). Glial fibrillary acidic protein (GFAP) is an intermediate-filament (IF) protein that is highly specific for cells of astroglial

lineage. Mutations identified in the GFAP gene have been related to the Alexander disease which is a rare disorder of the central nervous system (Brenner *et al.*, 2001).

LIM domain only 2 (rhombotin-like 1) (NM_005574). The protein encoded by this gene has a central role in hematopoietic development and is highly conserved (Yamada *et al.*, 1998).

Tetraspan 3 (NM_005724). This gene encodes a cell surface protein member of the tetraspanin family. The protein mediates signal transduction events that play a role in the regulation of cell development, activation, growth and motility (Berditchevski, 2001).

CD36 antigen (NM_005506). The protein encoded by this gene is a lysosomal integral membrane glycoprotein. Studies of a similar protein in mice and rats suggested that this protein may participate in membrane transportation and the reorganization of endosomal/lysosomal compartment (Calvo *et al.*, 1995).

Proteasome (prosome, macropain) subunit, alpha type, 3 (NM_002788). The proteasome is a multicatalytic protease complex that catalyzes an energy-dependent, extralysosomal proteolytic pathway responsible for selective elimination of proteins with aberrant structures and naturally occurring short-lived proteins related to metabolic regulation and cell cycle progression (Akioka *et al.*, 1995).

High-mobility group (nonhistone chromosomal) protein 2 (NM_002129). Studies suggest a role for this protein in facilitating cooperative interactions between cis-acting proteins by promoting DNA flexibility (Shirakawa & Yoshida, 1992).

STIP1 homoiogy and U-Box containing protein 1 (NM_005861). The protein encoded by this gene positively regulates parkin E3 activity. Parkin E3 is a ubiquitin-ligating enzyme involved in autosomal recessive juvenile Parkinsonism (Imai *et al.*, 2002).

Histidine triad nucleotide binding protein 1 (U51004). The protein encode by this gene is a member of a family of small proteins found in several species that inhibit PKC activity in the presence of the 14-3-3 proteins (Pearson *et al.*, 1990).

Hypothetical protein MGC8721 (NM_016127). Unknown function.

Myeloid/lymphoid or mixed-lineage leukemia 2 (NM_003482). The use of the human cDNA as a probe demonstrated that the gene is interrupted in both infant and adult acute myeloid (AML) and lymphoid (ALL) leukemia patients with 11q23 translocations (Parry *et al.*, 1993).

IGF-II mRNA-binding protein 1 (NM_006546). This gene encodes a member of the IGF-II mRNA-binding protein (IMP) family. The protein encoded by this gene contains four K homology domains and two RNA recognition motifs. Its function is to regulate IGF2 mRNA translation (Nielsen *et al.*, 1999).

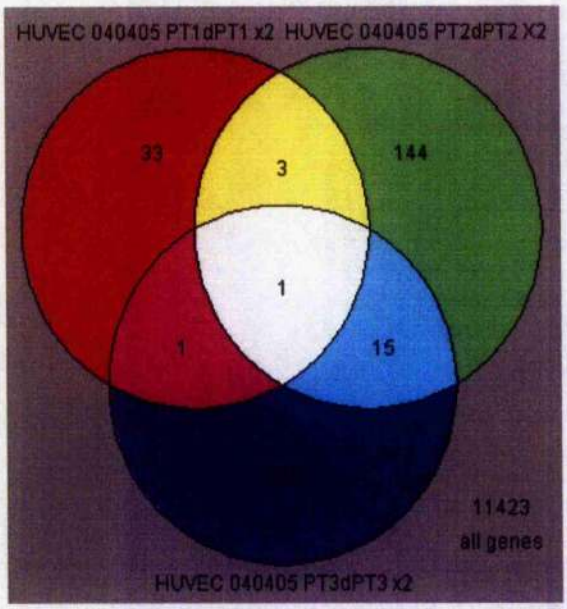


Figure 11. Up-regulated genes of interest selected from HUVEC microarray experiments

Venn diagram (left) and table (below). According to a colour code, the gene numbers from the diagram are associated with their name listed in the table. The gene lists analysed in the Venn diagram correspond to the lists of genes up-regulated by PT treatment minus the lists of genes up-regulated by detoxified PT (dPT) treatment, in experiment 1 (left circle), experiment 2 (right circle) and experiment 3 (bottom circle). The white section represents the number of genes up-regulated by PT only and present in all 3 experiments. The yellow section represents the number of genes up-regulated by PT only and present in 2 experiments: experiments 1 and 2. The light blue section represents the number of genes up-regulated by PT only and present in 2 experiments: experiments 2 and 3. The pink section represents the number of genes up-regulated by PT only and present in 2 experiments: experiments 1 and 3. The others sections correspond to the number of genes up-regulated by PT only and present in one experiment only: red (experiment 1), green (experiment 2) and blue (experiment 3). Coordinate is the location of the gene on the array.

Color code	Coordinate	Genbank access n°	Gene name
	P19ab3	NM_000742	cholinergic receptor, nicotinic, alpha polypeptide 2 (neuronal)
	D17ab7	NM_005567	lectin, galactoside-binding, soluble, 3 binding protein
	M21ef2	NM_016103	GTP-binding protein Sara
	O01ab4	NM_001383	diphthamide biosynthesis protein 2, s. cerevisiae, homolog-like 1
	P15gh5	NM_022760	chromosome 20 open reading frame 81
	C01ab4	NM_001260	cyclin-dependent kinase 8
	C21gh7	NM_021063	H2B histone family, member B
	E04cd6	NM_006086	tubulin, beta, 4
	E23ab5	NM_002055	glial fibrillary acidic protein
	F05ab7	NM_005574	LIM domain only 2 (rhombotin-like 1)
	F06cd5	NM_005724	tetraspan 3
	I22ab5	NM_005506	CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 2
	J11ab8	NM_002788	proteasome (prosome, macropain) subunit, alpha type, 3
	K04gh6	NM_002129	high-mobility group (nonhistone chromosomal) protein 2
	M03cd6	NM_005861	STIP1 homology and U-Box containing protein 1
	M03ef6	U51004	histidine triad nucleotide binding protein 1
	M14gh6	NM_003521	H2B histone family, member E
	N11ef2	NM_016127	hypothetical protein MGC8721
	O16cd3	NM_00348	myeloid/lymphoid or mixed-lineage leukemia 2
	P05cd6	NM_006546	IGF-II mRNA-binding protein 1

3.2.3.1.2. Down-regulation

EXPERIMENT 1/ EXPERIMENT 2/ EXPERIMENT 3

Fig. 12 presents in a Venn diagram the genes down-regulated by PT treatment only. The white section of the Venn diagram corresponds to the genes down-regulated by PT only and found in all three independent experiments. None of the genes passed this filter.

EXPERIMENT 1/ EXPERIMENT 2

In Fig. 12, two genes down-regulated after PT treatment only and found in experiments 1 and 2 are located in the yellow section. Those two genes are:

G protein-coupled receptor kinase 6 (NM_002082). Phosphorylation by receptor-specific and second messenger-activated protein kinases is a mechanism for regulation of G protein-coupled receptors. G protein-coupled receptors are 7-transmembrane domain-containing proteins and are triggered by a variety of signals. Gainetdinov *et al.* (2003) found that GRK6 is expressed in striatal neurons receiving dopaminergic input, and that postsynaptic D2/D3 dopamine receptors are physiological targets of this kinase.

Brain expressed, X-linked 1 (NM_018476). This human gene is mapped to the X chromosome and has an expression pattern of a spermatogenesis-related gene (Yang *et al.*, 2002).

EXPERIMENT 2/ EXPERIMENT 3

One gene downregulated after PT treatment was found in experiment 2 and 3:

CGI-141 protein (NM_016072). This gene encodes the protein Golgi transport 1 homologue B which has been reported to be positively regulated by I-kappaB kinase/NF-kappaB cascade (Matsuda *et al.*, 2003).

EXPERIMENT 1/ EXPERIMENT 3

The pink section of the Venn diagram corresponds of the genes down-regulated by PT only and commonly found in experiments 1 and 3. None of the genes passed this filter.

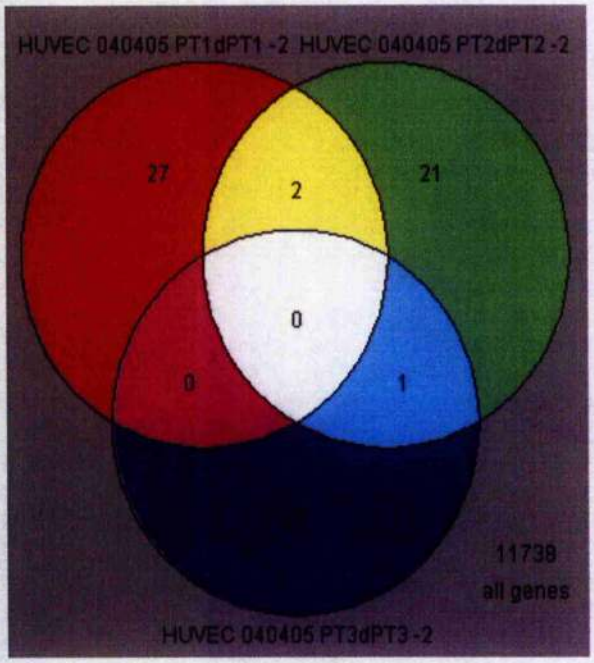


Figure 12. Down-regulated genes of interest selected from HUVEC microarray experiments
Venn diagram (left) and table (below). According to a colour code, the gene numbers from the diagram are associated with their name listed in the table. The gene lists analysed in the Venn diagram correspond to the lists of genes down-regulated by PT treatment minus the lists of genes down-regulated by detoxified PT (dPT) treatment, in experiment 1 (left circle), experiment 2 (right circle) and experiment 3 (bottom circle). The white section represents the number of genes down-regulated by PT only and present in 3 experiments. The yellow section represents the number of genes down-regulated by PT only and present in 2 experiments: experiments 1 and 2. The light blue section represents the number of genes down-regulated by PT only and present in 2 experiments: experiments 2 and 3. The pink section represents the number of genes down-regulated by PT only and present in 2 experiments: experiment 1 and 3. The others sections correspond to the number of genes down-regulated by PT only and present in one experiment only: red (experiment 1), green (experiment 2) and blue (experiment 3).

Color code	Coordinate	Genbank access n°	Gene name
	I08ab4	NM_002082	G protein-coupled receptor kinase 6
	P10gh4	NM_018476	brain expressed, X-linked 1
	A02ef2	NM_016072	CGI-141 protein

3.2.3.2. Effect of PT on gene expression of NL20 cells.

3.2.3.2.1. Up-regulation

EXPERIMENT 1/ EXPERIMENT 2

Fig. 13 presents in a Venn diagram the genes up-regulated by PT treatment only. The white section of the Venn diagram corresponds to the genes up-regulated by PT

only and they were identified in both independent experiments. Four genes passed this filter:

Leucine zipper, down-regulated in cancer 1 (NM_012317). The protein (LDOC1) encoded by this gene is localized to the nucleus and is down-regulated in some cancer cell lines. Observations suggest that LDOC1 is a novel regulator of NF-kappa β that can affect the TNF-alpha-mediated pathway to apoptosis through inhibition of NF-kappa β activation in BxPC3 pancreatic cancer cells (Nagasaki *et al.*, 2003).

Ubiquitin-conjugating enzyme E2 variant 1 (NM_003349). The gene product could be involved in poly-ubiquitin chain synthesis (Hershko & Ciechanover, 1998).

G-protein-coupled receptor 41 (NM_005304). The protein encoded by the gene is characterized, in human tissue, as a receptor for short chain fatty acids (Le Poul *et al.*, 2003).

Chlorine channel 3 (NM_001829). Studies showed that CLCN3 is an intracellular chloride channel. It is also present on synaptic vesicles, where it contributes to their acidification (Stobrawa *et al.*, 2001).

EXPERIMENT 1

The pink section of Fig. 13 is related to the genes up-regulated after PT treatment only. This gene list was generated from experiment one and contains 311 genes.

In order to present the more relevant genes from this list, the option of GeneSpring software named Gene ontology (GO) was used. This software groups genes hierarchically into meaningful biological categories. According to the aims of the project, only those genes related to cell-cell signalling, host pathogen interaction, response to external stimulus or cell recognition were selected. The gene list contained 8 genes:

CD44 antigen (M59040). The protein encoded by this gene is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. It is a receptor for hyaluronic acid (HA) and can also interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs) (Brown *et al.*, 2005).

CD63 antigen (NM_001780). The protein encoded by this gene is a member of the transmembrane 4 superfamily, also known as the tetraspanin family. This encoded protein is a cell surface glycoprotein that is known to complex with integrins. It may function as a blood platelet activation marker. Deficiency of this protein is associated with Hermansky-Pudlak syndrome (Zhang *et al.*, 2003). The Hermansky-Pudlak syndrome (HPS) is an autosomal recessive disorder. It consists of a triad that includes the following: tyrosinase-positive oculocutaneous albinism (Ty-pos OCA), bleeding diathesis, and systemic complications associated to ceroid-lipofuscin–like lysosomal storage disease.

Insulin growth factor binding protein 3 (NM_000598). This gene is a member of the insulin-like growth factor binding protein family. The protein binds insulin-like growth factor (IGF) I or II. In this form, it circulates in the plasma, prolonging the half-life of IGFs and altering their interaction with cell surface receptors (Kiefer *et al.*, 1991).

Platelet-derived growth factor beta polypeptide (X02811). The protein encoded by this gene is a mitogenic factor for cells of mesenchymal origin. Mutations in this gene are associated with meningioma (Bolger *et al.*, 1985).

Tumor necrosis factor receptor superfamily (NM_003844). The protein encoded by this gene is a member of the TNF-receptor superfamily. This receptor is activated by tumor necrosis factor-related apoptosis inducing ligand (TNFSF10/TRAIL) and thus transduces a cell death signal and induces cell apoptosis (Pan *et al.*, 1997).

Thy-1 cell surface antigen (NM_006288). Thy-1 is the designation for a major cell surface glycoprotein characteristic of T cells. A study identify the gene Thy-1 associated with tumor suppression in the ovarian cell line SKOV-3 (Abeysinghe *et al.*, 2003).

Vascular endothelial growth factor C (U43142). The protein encoded by this gene is a member of the platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) family. It is active in angiogenesis and endothelial cell growth, and can also affect the permeability of blood vessels (Joukov *et al.*, 1996).

Diphthamide biosynthesis protein 2 (NM_001383). See section 3.2.4.1. Effect of PT on gene expression of HUVEC primary cells. Section 3.2.4.1.1. Up-regulation. Array 1/2/3.

EXPERIMENT 2

The light blue section of Fig. 13 shows the genes up-regulated after PT treatment only. This gene list is generated from experiment two only and contains 125 genes. For experiment 2, genes related to cell-cell signalling, host pathogen interaction, response to external stimulus or cell recognition were selected. The resulting list contained 6 genes:

Adenylate cyclase-activating polypeptide 1 (pituary) receptor type 1 (NM_001118). This gene encodes type I adenylate cyclase activating polypeptide receptor, which is a membrane-associated protein and shares significant homology with members of the glucagon/secretin receptor family. This receptor mediates diverse biological actions of adenylate cyclase-activating polypeptide 1 and is positively coupled to adenylate cyclase. The type 1 receptor, which is found in the hypothalamus, brainstem, pituitary, adrenal gland, pancreas, and testes, has a high

affinity only for PACAP (pituitary adenylate cyclase-activating polypeptide) (Ogi *et al.*, 1993).

B-cell receptor-associated protein BAP29 (NM_018844). The protein encoded by this gene could be involved in apoptosis (Strausberg *et al.*, 2002).

Cholinergic receptor, nicotinic, delta polypeptide (NM_000751). The acetylcholine receptor of muscle has 5 subunits of 4 different types: 2 alpha and 1 each of beta, gamma and delta subunits. After acetylcholine binding, the receptor undergoes an extensive conformation change that affects all subunits and leads to opening of an ion-conducting channel across the plasma membrane (Miyazawa *et al.*, 2003).

Small inducible cytokine subfamily A (Cys-Cys) Member 20 (NM_003250). CCL20 chemokine encoded by this gene has negligible activity toward neutrophils, monocytes, and naive T lymphocytes. However, flu antigen plus IL2 -activated CD4+ and CD8+ T lymphoblasts and cord blood-derived dentritic cells responded to both forms of CCL20 (Nelson *et al.*, 2001).

Thyroid hormone receptor, alpha (NM_003250). The protein encoded by this gene is a nuclear hormone receptor for triiodothyronine (T3)(DeLong *et al.*, 2004).

CD36 antigen-like 2 (NM_005506). See section 3.2.4.1. Effect of PT on gene expression of HUVEC primary cells. Section 3.2.4.1.1. Up-regulation. Experiments 2/3.

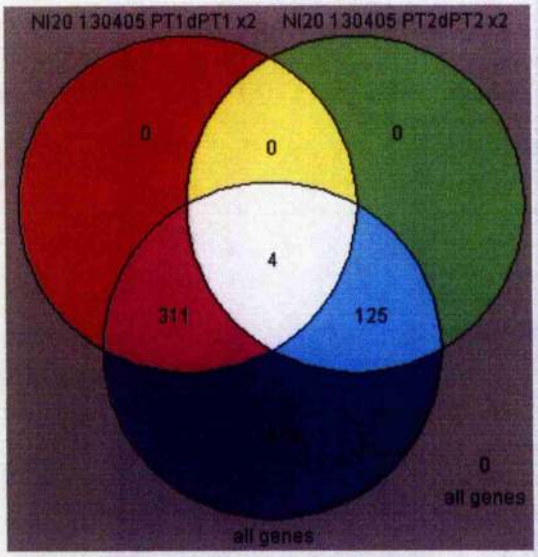


Figure 13. Up-regulated genes of interest selected from NL20 microarray experiments

Venn diagram (left) and table (below). According to a colour code, the gene numbers from the diagram are associated with their name listed in the table. The gene lists analysed in the Venn diagram correspond to the lists of genes up-regulated by PT treatment minus the lists of genes up-regulated by detoxified PT (dPT) treatment, in experiment 1 (left circle) and experiment 2 (right circle). The bottom circle shows genes investigated in each experiment (Total 11856 genes). The white section represents the number of genes up-regulated by PT only and present in 2 experiments. The pink section represents the number of genes up-regulated by PT only and present in experiment 1 only. The light blue section represents the number of genes up-regulated by PT only and present in experiment 2 only. Genes listed in the table related to light blue and pink colours were selected by Genespring software as member of the cell-cell signalling, host pathogen interaction, response to external stimulus or cell recognition gene ontologies.

Color code	Coordinate	Genbank access n°	Gene name
	G01cd8	NM_012317	leucine zipper, down-regulated in cancer 1
	O23cd3	NM_003349	ubiquitin-conjugating enzyme E2 variant 1
	G02ab6	NM_005304	G protein-coupled receptor 41
	I02ab5	NM_001829	chloride channel 3
	O21ef1	M59040	CD44 antigen
	O18ab4	NM_001780	CD63 antigen
	D07ab6	NM_000598	insulin-like growth factor binding protein 3
	A08ef5	x02811	platelet-derived growth factor beta polypeptide
	G03cd4	NM_003844	tumor necrosis factor receptor superfamily
	o01ab4	NM_001383	diphthamide biosynthesis protein 2, s. cerevisiae, homolog-like
	B02cd2	NM_006288	thy-1 cell surface antigen
	008ef7	U43142	vascular endothelial growth factor C
	I15ab2	NM_001118	adenylate cyclase activating polypeptide 1(pituitary) receptor
	J18gh4	NM_018844	B-cell receptor-associated protein BAP29
	I22ab5	NM_005506	CD36 antigen-like 2
	B10ab3	NM_000751	cholinergic receptor, nicotinic, delta polypeptide
	m02cd2	NM_004591	small inducible cytokine subfamily A (Cys-Cys), member 20
	k20gh6	NM_003250	thyroid hormone receptor, alpha

3.2.3.2.2. Down-regulation

EXPERIMENT 1/ EXPERIMENT 2

Fig. 14 presents in a Venn diagram the genes down-regulated by PT treatment only. The white section of the Venn diagram corresponds to genes down-regulated by PT only and found in both independent experiments. None of the genes passed the filter.

EXPERIMENT 1

According to Fig. 14, 77 genes down-regulated after PT treatment only were found in experiment 1 (pink section). Using the gene ontology option of GeneSpring software, genes related to cell-cell signalling, host pathogen interaction, response to external stimulus or cell recognition were selected. The resulting list contained 6 genes:

Opioid growth factor receptor (NM_007346). The protein encoded by this gene is a receptor for opioid growth factor (OGF) which is a negative regulator of cell proliferation and tissue organization during development, cellular renewal, cancer, wound healing, and angiogenesis (Zagon *et al.*, 2000).

Thyroid hormone receptor interactor 12 (NM_004238). The thyroid hormone receptors (TRs) are hormone-dependent transcription factors that regulate expression of a variety of specific target genes. They must specifically interact with a number of proteins as they progress from their initial translation and nuclear translocation to heterodimerization with retinoid X receptors (RXRs), functional interactions with other transcription factors and the basic transcriptional apparatus, and eventually, degradation (Lee *et al.*, 1995).

Teratocarcinoma-derivated growth factor 3, pseudogene (M96956). The TDGF3 locus has characteristics of a retrotransposon, including lack of introns and a poly(A) sequence (Dono *et al.*, 1991).

Cancer/testis antigen1 (NM_001327). Molecular function and biological process unknown (Chen *et al.*, 1997).

Immunoglobin heavy constant epsilon (J00222). This gene encodes immunoglobulins of the IgE isotype are responsible for the immediate hypersensitivity reactions that occur in diseases such as hay fever, allergic asthma, and anaphalaxis (Nishida *et al.*, 1982).

KH domain containing, RNA binding, signal transduction associated 1 (NM_006559). The protein encoded by this gene (SAM68) is a tyrosine-phosphorylated, Src-associated protein in mitotic cells. The protein SAM68 binds to DNA and mRNA SAM68 and may have a role in cell cycle control, particularly at the G1/S transition (Barlat *et al.*, 1997).

EXPERIMENT 2

According to Fig. 14, 69 genes downregulated after PT treatment only are found in experiment 2 (light blue section). Using the gene ontology option of GeneSpring software, genes related with cell-cell signaling, host pathogen interaction, response to external stimulus or cell recognition were selected. The resulting list contains 4 genes:

Solute carrier family 6 (NM_004211). The amino acid glycine is a major inhibitory neurotransmitter in the spinal cord, brainstem, and retina, where it exerts its effects on the strychnine-sensitive glycine receptors (Morrow *et al.*, 1998).

CD79A antigen (Immunoglobulin-associated alpha) (NM_001783). The B lymphocyte antigen receptor is a multimeric complex which includes the antigen-specific component, surface immunoglobulin (Ig). This gene encodes the Ig-alpha protein of the B-cell antigen component (Reth, 1992).

Integrin, alpha 4 (L12002). Integrins are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain. Alpha 4 combines with beta 1 (ITGB1) on T-cells to form the integrin very late (activation) antigen 4 (VLA-4) that can bind to the extracellular matrix molecules fibronectin or thrombospondin, and is also a ligand for the cell surface molecule vascular cell adhesion molecule 1 (VCAM-1) (Cunningham *et al.*, 2002).

Lymphocyte cytosolic protein 2 (NM_005565). The protein encoded by this gene plays a positive role in promoting T cell development and activation as well as mast cell and platelet function (Motto *et al.*, 1996).

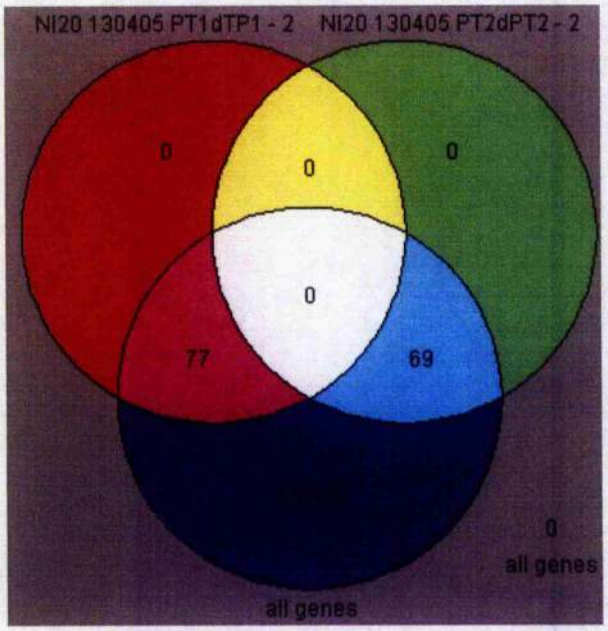


Figure 14. Down-regulated genes of interest selected from NL20 microarray experiments
Venn diagram (left) and table (below). According to a colour code, the gene numbers from the diagram are associated with their name listed in the table. The gene lists analysed in the Venn diagram correspond to the lists of genes down-regulated by PT treatment minus the lists of genes down-regulated by detoxified PT (dPT) treatment, in experiment 1 (left circle) and experiment 2 (right circle). The bottom circle shows genes investigated in each experiment (Total 11856 genes). The white section represents the number of genes down-regulated by PT only and present in 2 experiments. The pink section represents the number of genes down-regulated by PT only and present in experiment 1 only. The light blue section represents the number of genes down-regulated by PT only and present in experiment 2 only. Genes listed in the table related to light blue and pink colours were selected by GeneSpring software as member of the cell-cell signaling, host pathogen interaction, response to external stimulus or cell recognition gene ontologies.

Color code	Coordinate	Genbank access n°	Gene name
	F03cd7	NM_00734	opioid growth factor receptor
	K05gh1	NM_00423	thyroid hormone receptor interactor 12
	O04ef7	M96956	teratocarcinoma-derived growth factor 3, pseudogene
	O03ab6	NM_00132	cancer/testis antigen 1
	E15ef1	J00222	immunoglobulin heavy constant epsilon
	b04cd6	NM_00655	KH domain containing, RNA binding, signal transduction
	f14cd4	NM_00421	solute carrier family 6
	p03ab5	NM_00178	CD79A antigen (immunoglobulin-associated alpha)
	e19ef7	L12002	integrin, alpha 4
	j15cd7	NM_00556	lymphocyte cytosolic protein 2

3.3. RT-PCR

All cDNAs were produced by semi-quantitative RT-PCR as follow explained: Total RNA samples from control and toxin-treated cells, were diluted and reverse transcribed using OligodT primers. The resulting cDNAs were then used for PCR amplification with specific primers (Table 3), depending on the gene under investigation. In each experiment, the glyceraldehyde-3-phosphate dehydrogenase

(GapDH) gene was used as an internal control. For each experiment, the amount of signal was recorded and normalised with reference to the signal obtained for the housekeeping gene. Ratios of level of expression were calculated from the lowest amount of RNA that gave a signal.

3.3.1. HUVEC cells

3.3.1.1. Confirmation of microarray results by semi-quantitative RT-PCR

From the HUVEC microarray experiments, four genes candidates were selected as potential markers of PT toxicity. The cholinergic receptor gene (CHRNA) was found to be up-regulated specifically by PT in three independent experiments. Three other genes were selected because, at the time that primer design was performed, microarray results were available only in duplicate. Therefore, these genes showed specific up-regulation by PT in experiments 1 and 2. They were: galectin 3 binding protein (galectin 3), diphthamide biosynthesis protein 2 homolog-like 1 (DPH2L1) and SAR1a gene homolg 2 (SAR1). A further gene investigated in RT-PCR experiments was the cAMP response element-binding protein (CREBp1) gene. This gene was reported to be specifically up-regulated by the enzyme activity of PT in a microarray study carried out on BEAS-2B cells (Belcher *et al.*, 2000). This gene was used in order to attempt to reproduce published data, where a different microarray protocol had been used.

The aim of the semi-quantitative RT-PCR experiments was to confirm the data obtained by the microarray techniques. Fig. 15 shows agarose gels of PCR amplification products that were generated from three different sets of RNA samples related to experiment 1-3. The ratios of expression level were calculated for each gene amplified and are presented in Table 6. From the three independent experiments (experiments 1, 2 and 3), only RT-PCR performed from RNA samples

from experiment 2 showed signals at the expected size for four of the targetted genes: CREBp1, Galectin 3, DPH2L1 and SAR1. However, all the ratios of the expression level were less than 2: 1.79, 1.87, 1.97 and 1.39 for CREBp1, Galectin 3, DPH2L1 and SAR1 respectively. Taking into account that a) only one of the three cDNA preparation gave significant signals and b) ratios of expression level calculated from microarray experiments 1, 2 and 3 were (0; 0; 0) for CREBp1, (2.56; 15.8; 0) for Galectin 3, (5.9; 2; 0) for DPH2L1, (9.3; 2.4; 2.3) for CHRNA, and (2.52; 53.3; 0) for SAR1. Therefore we concluded that the data produced using the microarray technique could not be confirmed by RT-PCR.

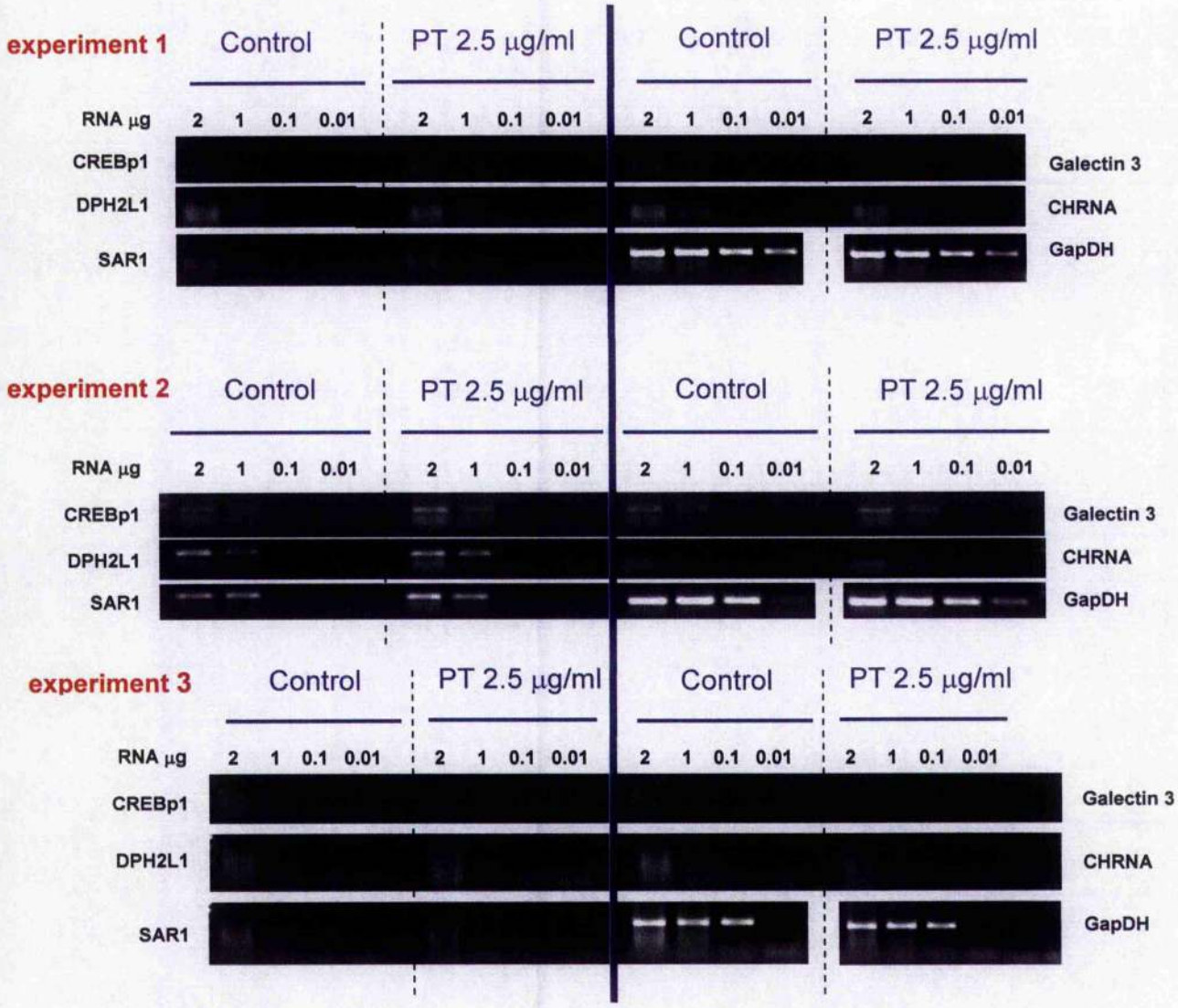


Figure 15. Semi-quantitative RT-PCR products from genes as possible markers of PT toxicity from HUVEC microarray experiments.

Total RNA was extracted from HUVEC cells ($5x10^5$ cells/ml) that had been treated with PT (2.5 µg/ml) for 6h. Total RNA was diluted (2, 1, 0.1 and 0.01µg/gene) and reverse transcribed into cDNA. Then PCR was carried out using primers to specifically amplify 6 genes: CREBp1, Galectin 3, DPH2L1, CHRNA, SAR1 and GapDH. After 30 cycles of amplification and a final step of elongation at 72^0C for 7 min, the PCR products (Control and PT treatment) for each gene tested, were loaded in a 1.2% agarose gel containing ethidium bromide and electrophoresed for 1 h. Three sets of RT-PCR products are presented in Fig. 15. They correspond to RNA used for microarray experiments 1-3. Only RT-PCR products from experiment 2 showed signals at the expected size (~ 500 bp) for the gene candidate CREBp1, Galectin 3, DPH2L1 and SAR1. The lowest amounts of RNA samples that gave detectable signals were used for calculation of the expression of genes relative to the control group (See Table 5). GapDH gene was used as an internal control.

Table 6. Comparative analysis of expression level ratios obtained by microarrays and semi quantitative RT-PCR on HUVEC and NL20 cells

Cell type	Experiment type	Treatment	CREBp1	Galectin 3	DPH2L1	CHRNA	SAR1	ICAM-1	VCAM-1	E-selectin
HUVEC	MICRO ARRAY	PT 2.5 µg/ml 6 h experiment 1	0	2.56	5.9	9.3	2.52	0	0	
		PT 2.5 µg/ml 6 h experiment 2	0	15.8	2	2.4	53.3	0	0	
		PT 2.5 µg/ml 6 h experiment 3	0	0	0	2.3	0	0	0	
	RT-PCR	PT 2.5 µg/ml 6 h experiment 1	0	0	0	0	0			
		PT 2.5 µg/ml 6 h experiment 2	1.79	1.87	1.97	0.6	1.39			
		PT 2.5 µg/ml 6 h experiment 3	0	0	0	0	0			
		PT 100ng/ml 18 h + IL1 beta (5 IU/ml) 4 h	0	0	0	0	0	0.7	0.2	0.6
		PT 100 ng/ml 18 h + TNF alpha (20 IU/ml) 6 h		0			0	5	1.5	1.3
NL20	MICRO ARRAY	PT 2.5 µg/ml 6 h experiment 1	0							
		PT 2.5 µg/ml 6 h experiment 2	0							
	RT-PCR	PT 2.5 µg/ml 6 h experiment 1	0							
		PT 2.5 µg/ml 6 h experiment 2	0.91							

The ratios of gene expression relative to controls are given as numbers. When no signal was recorded by microarray or RT-PCR assessments 0, was recorded in the table. If the gene was not investigated in the experiment the box was coloured in grey. The amount of signal for each band in the gels was measured and normalised according to the signal for the GapDH gene. Finally, the lowest amounts of RNA samples that gave detectable signals, were used for calculation of the expression of genes relative to the control.

3.3.1.2. Effect of PT and IL-1 β on HUVEC cells.

In an attempt to get closer to *in vivo* conditions and to increase the sensitivity of signal detection, PT treatments in association with IL-1 beta were carried out on HUVEC cells. Four different groups of cells were treated:

Group 1: for 22 h with medium alone

Group 2: for 18 h with medium alone + for 4 h with IL-1β (5 IU/ml)

Group 3: for 18 h with PT (100 ng/ml) + for 4 h with medium alone

Group 4: for 18 h with PT (100 ng/ml) + for 4 h with IL-1β (5 IU/ml).

Fig. 16 shows results obtained from a semi-quantitative RT-PCR carried out on total RNA extracted from HUVEC cells treated for 18 h with PT (100 ng/ml) and further stimulated with IL-1 β (5 IU/ml) for 4 h. Genes investigated in this experiment were the five genes selected as potential markers of PT toxicity: CREBp1, Galectin 3, DPH2L1, CHRNA and SAR1. No signal was detected for 4 genes: CREBp1, Galectin 3, CHRNA and SAR1 (Fig. 16). Signals at the expected size were observed for the DPH2L1 gene. RNA samples from the control Group 2 (medium only + IL-1 β) showed signals for an amount of RNA of 2 and 1 µg. On the other hand, RNA samples from Group 4 (PT+IL-1 β) showed signals for an amount of RNA of 1 µg only (See Figure 16 a). The signal was not consistent for all the RNA amounts tested; therefore the ratio of expression level could not be calculated (Table 6). Taking this information together, PT treatment in association with IL-1 β stimulation did not induce an up-regulation of gene expression of the candidate genes tested.

Figure 16 a. Semi-quantitative RT-PCR products of selected genes expressed by HUVEC cells after treatment with PT and IL-1 β

Total RNA was extracted from HUVEC cells ($5x10^5$ cells/ml) that were untreated or had been treated with PT (100 ng/ml) for 18 h and further stimulated with IL-1 β (5 IU/ml) for 4 h. Total RNA was diluted (2, 1, 0.1 and 0.01µg/gene) and reverse transcribed into cDNA. Then, PCR was carried out using primers to specifically amplify six genes: CREBp1, Galectin 3, DPH2L1, CHRNA, SAR1 and GapDH. After 30 cycles of amplification and a final step of elongation at 72^0C for 7 min, the PCR product (Control media + IL-1 β stimulation and PT treatment + IL-1 β stimulation) for each gene tested, were loaded in a 1.2% agarose gel containing ethidium bromide and electrophoresed for 1h.

A second semi-quantitative RT-PCR experiment was carried out in order to investigate the effect of PT treatment in association with IL-1 β stimulation on the gene expression of five genes. The results of this experiment are illustrated in Fig. 16 b. Two of the genes were candidate for markers of PT toxicity: Galectin 3 and DPH2L1. The other three genes code for adhesion molecules: ICAM-1, VCAM-1 and E-selectin. Neither of the candidate genes for toxicity showed signals at any RNA amount tested, thus confirming that PT treatment, in association with IL-1 β stimulation, at the time points and concentrations used, did not induce an increase in gene expression.

The genes ICAM-1, VCAM-1 and E-selectin show signals at the expected size (~250 bp). The three genes encoding adhesion molecules were amplified at 1 and 0.1µg of RNA samples for ICAM-1 and E-selectin and at 1 to 0.01 µg of RNA sample for VCAM-1 (See Figure 16 b). The amount of signal for each band was recorded and normalised with the signal recorded for the GapDH gene. The value obtained for the

lowest amounts of RNA samples after PT+IL-1 β (Group 4) treatment were divided by the value obtained for the lowest amounts of RNA sample from the control+IL-1 β (Group 2). Table 6 presents the ratios of expression levels calculated. The ratios are 0.7, 0.2 and 0.6 for ICAM-1, VCAM-1 and E-selectin respectively. The amounts of the signal produced by amplification of GapDH are lower for PT+IL-1 β treatment than for the controls + IL-1 β. Thus, data produced in this experiment show an apparent down-regulation of gene expression.

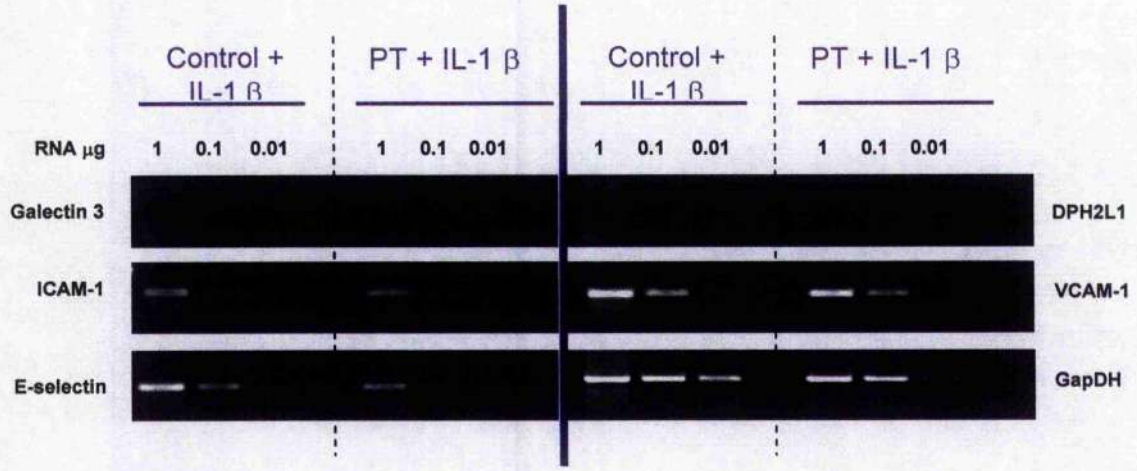


Figure 16 b. Semi-quantitative RT-PCR products of further selected genes expressed by HUVEC cells after treatment with PT and IL-1 β

Total RNA was extracted from HUVEC cells ($5x10^5$ cells/ml) that were untreated or had been treated with PT (100 ng/ml) for 18 h and further stimulated with IL-1 β (5 IU/ml) for 4 h. Total RNA was diluted (1, 0.1 and 0.01µg/gene) and reverse transcribed into cDNA. Then the PCR was carried out using primers to specifically amplify six genes: Galectin 3, DPH2L1, ICAM-1, VCAM-1, E-selectin and GapDH. After 30 cycles of amplification and a final step of elongation at 72^0C for 7 min, the PCR product for each gene tested, were loaded in a 1.2% agarose gel containing ethidium bromide and electrophoresed for 1 h.

3.3.1.3. Effect of PT and TNF α on HUVEC cells.

For the same purpose as for IL-1 β treatment (3.3.1.2), PT treatments in association with TNF α were carried out on HUVEC cells. Four different groups of cells were treated:

Group 1: for 24h with medium alone

Group 2: for 18h with medium alone + for 6 h with TNFα (20 IU/ml)

Group 3: for 18h with PT (100 ng/ml) + for 6 h with medium alone

Group 4: for 18h with PT (100ng/ml) + for 6h with TNFα (20 IU/ml).

Figure 17 shows the results obtained from a semi-quantitative RT-PCR carried out on total RNA extracted from HUVEC cells treated for 18 h with PT (100 ng/ml) and further stimulated with TNF α (20 IU/ml) for 6 h. Five genes were investigated in this experiment, two of them were candidate genes as markers of PT toxicity: Galectin 3

and SAR1. Three other genes encode adhesion molecules: ICAM-1, VCAM-1 and E-selectin. No signal was detected for the two gene candidates of PT toxicity: Galectin 3 and SAR1. Therefore, it was concluded that PT treatment in association with TNF α stimulation did not increase the sensitivity of the signal detection of the candidate genes tested in this experiment.

ICAM-1, VCAM-1 and E-selectin genes showed signals at the expected size (~250 bp) (Fig. 17). The E-selectin gene was amplified at 1 µg of RNA sample in the treatment groups 1, 2, 3 and 4. The gene was also amplified at 0.1 µg of RNA sample in the group 4. The ICAM-1 gene was amplified at 1 µg of RNA sample in the groups 1, 2, 3 and 4. The gene was also amplified at 0.1 µg of RNA sample in group 4. The VCAM-1 gene was amplified at 1 µg of RNA sample in groups 1, 2, 3 and 4. The gene was also amplified at 0.1 µg of RNA sample in groups 2 and 4. The amplification product observed at 0.1 µg of RNA sample in the group 1 was not at the expected size (259bp). Ratios of expression levels were calculated for these three genes between the normalised signals obtained for the treatment PT+TNF α (Group 4) and the treatment TNF α alone (Group 2). The ratios reported in Table 6 are 5, 1.5 and 1.3 for ICAM-1, VCAM-1 and E-selectin genes respectively. Thus, the treatment of PT in association with TNF α stimulation seems to increase the expression level of the ICAM-1 gene in HUVEC cells.

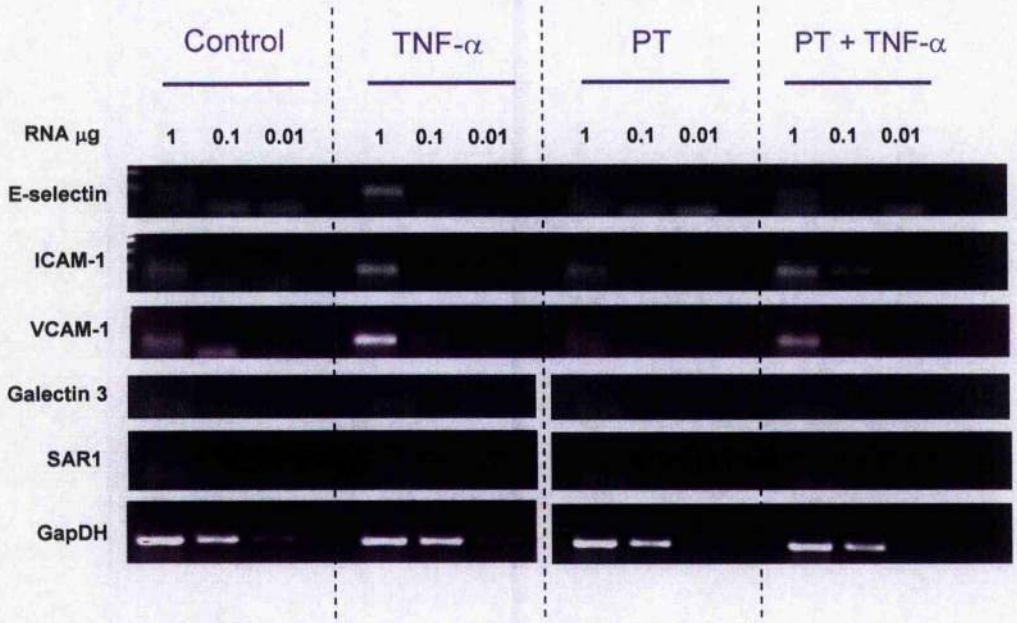


Figure 17. Semi-quantitative RT-PCR products of selected genes expressed by HUVEC cells after treatment with PT and TNF α

Total RNA was extracted from HUVEC cells ($5x10^5$ cells/ml) that were untreated or treated with PT (100 ng/ml) for 18 h and further stimulated with TNF α (20 IU/ml) for 6 h. Total RNA was diluted (1, 0.1 and 0.01 µg/gene) and reverse transcribed into cDNA. Then the PCR was carried out using primers to specifically amplify six genes: E-selectin, ICAM-1, V-CAM-1, Galectin 3, SAR1 and GapDH. After 30 cycles of amplification and a final step of elongation at 72^0C for 7 min, the PCR product for each gene tested, were loaded in a 1.2% agarose gel containing ethidium bromide and electrophoresed for 1 h.

3.3.2. NL20 cells

At the time that the RT-PCR experiments were started, the result of only one microarray experiment on NL20 cells were available. The number of genes specifically up-regulated by PT treatment was 471 and thus the selection of suitable candidate genes was difficult. Gene candidates had to be chosen and further analysed. The cAMP response element-binding protein (CREBp1) had been reported to be specifically up-regulated by the enzyme activity of PT in a microarray study carried out on BEAS-2B cells (Belcher *et al.*, 2000). Therefore only one gene, CREBp1, was investigated in order to try to reproduce published data that had been obtained using a different microarray protocol.

Fig. 18 shows agarose gels of the PCR amplification products generated from two different sets of RNA samples related to experiments 1 and 2. Primers were used to specifically amplify the CREBp1 and GapDH (housekeeping) genes. From the two independent experiments (experiments 1 and 2), only the RT-PCR performed from RNA samples related to microarray 2 showed signals at the expected size for the CREBp1 gene. The ratio of expression level was calculated for the lowest amount of RNA sample that gave a signal and is presented in Table 6. This ratio was 0.91. Thus, there was no evidence that the CREBp1 gene was up-regulated by PT treatment of NL20 cells and the results obtained in the Belcher *et al* (2000) study could not be confirmed.

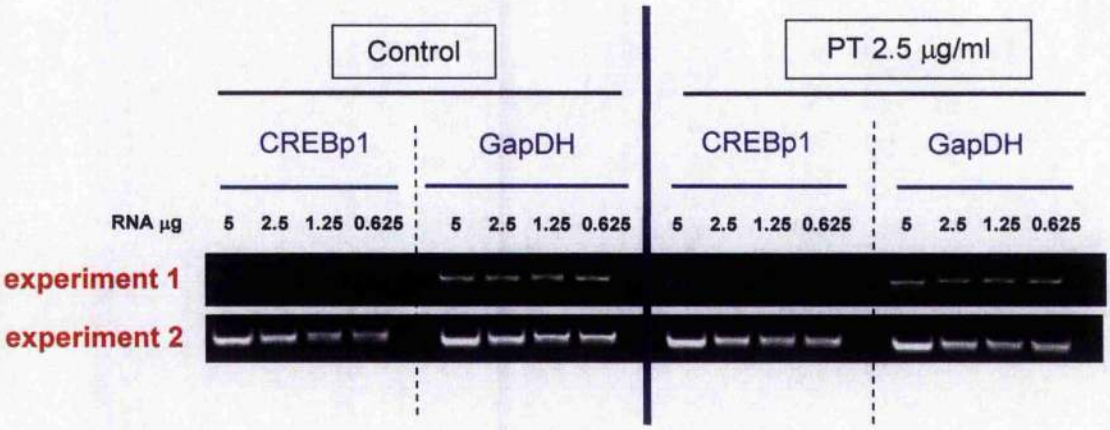


Figure 18. Semi-quantitative RT-PCR products of the CREBp1 gene expressed by NL20 cells after treatment with PT.

Total RNA was extracted from NL20 cells ($5x10^5$ cells/ml) that were untreated or had been treated with PT (2.5 µg/ml) for 6 h. Total RNA was diluted (5, 2.5, 1.25 and 0.625 µg/gene) and reverse transcribed into cDNA. Then the PCR was carried out using primers specific for the CREBp1 gene. After 30 cycles of amplification and a final step of elongation at 72^0C for 7 min, the PCR products were loaded in a 1.2% agarose gel containing ethidium bromide and electrophoresed for 1 h. Two sets of RT-PCR products are presented in Figure 18, they correspond to RNA used for experiments 1 and 2. Only experiment 2 RT-PCR products show signals at the expected size (~500 bp) for CREBp1. The lowest amounts of RNA samples that gave detectable signals were used for calculation of the expression of genes relative to the control group. GapDH was used as an internal control.

3.4. Cell-migration study

A study was carried out in order to investigate the potential effect of PT on endothelial cell migration. A chemotaxis chamber was used for this purpose. This was composed of 48 wells, each of them separated into two by a porous membrane coated with fibronectin (6.5 µg/ml). Cells seeded in the upper well were in contact by capillarity with the contents of the lower well. PT solutions at 2.5 µg/ml or 10 µg/ml, medium alone (negative control), LPS 100 ng/ml or FCS 10% v/v (positive controls) were added to the culture medium in the lower wells. After incubation for 4 h, the cells that had migrated into or through the membrane were counted and the ratios of counts in the treatment groups to that in the negative control group were calculated to allow comparison (Table 6). The ratios between the number of migrated cells in the positive (LPS or FCS) and the negative control were also calculated. According to the results presented in Table 6, LPS seems to be the most appropriate positive control for the cell-migration assay. In 7 of a total of 8 experiments, there was a marked difference between the LPS control and the negative control. With the FCS control, 2 out of 4 experiments did not show a difference from the negative control. Therefore, for interpretation of the results, data from the experiments which used LPS as positive control, and the ratios of positive/negative >1 were taken into consideration (Table 6). For PT treatment groups at concentrations of 10 µg/ml and 2.5 µg/ml, there were 6 out of 7 assays that showed the ratios to be >1 (1.8-2.9) and >1 (1.7-2.5), respectively. However, there was no clear dose-response either within the same assay or between assays (Table 6). The coefficient of variation for the assays were calculated. The coefficient of variation (CV%) is the standard deviation divided by the mean, and the result is given as a percentage. It should also be noted

that the assay variability was large; the CV% for the ratio of positive/negative was 43.9. Further optimisation of the assay conditions is needed. Nevertheless, the preliminary results indicated that PT at the concentrations used could induce the migration of endothelial cells after incubation for 4 h.

Table 7. Comparison of numbers of HUVEC cells migrating in response to PT treatment

Name of the assay	Cells migrated in negative control	Cells migrated in positive control	Ratio positive/ negative controls	Cells migrated in PT 10 µg/ml	Ratio PT 10 µg/ml / negative control	Cells migrated in PT 2.5 µg/ml	Ratio PT 2.5 µg/ml / Negative control
1	21.3	56.8 (LPS)	2.6	63	2.9	41	1.9
2	17.9	8.4 (LPS)	0.5	3	0.17	3	0.2
3	20	25 (LPS)	1.2	18	0.9	32	1.6
4	8	10 (LPS)	1.2	15	1.9	12.5	1.6
5	11	30 (LPS)	2.7	30	2.7	28	2.5
6	6	13 (LPS)	2.2	15	2.5	6	1
7	4	15 (LPS)	3.7	10	2.5	7	1.75
8	22.5	32.5 (LPS)	1.4	40	1.8	37.5	1.7
9	70	80 (FCS)	1	58	0.8	110	1.6
10	37	51 (FCS)	1.4	25	0.7	33	0.9
11	71	70 (FCS)	1	34	0.5		
12	9	14.4 (FCS)	1.6	6.8	0.7		

HUVEC cells ($5x10^4$ cells) were placed in the upper wells of the chemotaxis chamber. The bottom wells of the chamber contained 4 different treatments: negative control (Medium alone), PT at 10 µg/ml, PT at 2.5 µg/ml and positive controls (LPS at 100 ng/ml or FCS at 10% solutions). The upper and lower wells were separated by a porous membrane containing 8 µm pores to allow cell/treatment contact and migration. Cells were incubated for 4h. The table shows the number of cells that migrated into or through the membrane after staining. Ratios between treatments were calculated to allow comparison. If the positive/negative control ratio was <1, the data of the experiment were coloured in grey and not taken into account for interpretation. Ratios calculated by dividing the number of migrated cells in the negative control by the number of migrated cells in the treatment are presented for PT 10 µg/ml and PT 2.5 µg/ml. In these two columns when the ratio was >1, the box was coloured in red and when the ratio <1, the box was coloured in green.

4. DISCUSSION

Pertussis vaccines are commonly used both in the UK and worldwide for the prevention of the disease pertussis (whooping cough). Two types of pertussis vaccines, the whole-cell pertussis vaccines (WCV) and acellular pertussis vaccines (ACV), are recommended by WHO. The Histamine Sensitisation Test (HIST) is a test required by the European Pharmacopoeia and the WHO for the quality control of pertussis vaccines (European Pharmacopoeia 1997, pp 1305-1307, WHO Technical Series, Forty-seventh Report: pp57-76. 1998). It is designed specifically to control the toxicity of pertussis toxin (PT) in these vaccines. HIST is a lethal challenge test on animals and large variations in test performance have been observed. The procedure has proved very difficult to standardise. This often leads to a requirement for repeat tests and large numbers of animals are being used by vaccine manufacturers and control laboratories.

The aim of the present study was the identification of gene markers of pertussis toxin toxicity using the microarray technique, in order to develop *in vitro* assays based on human cell lines as an alternative to the HIST. The study had three main goals:

1) determination of a suitable concentration of PT for microarray experiments,

2) investigation of gene expression of HUVEC and NL20 cells after pertussis toxin and detoxified pertussis toxin treatments using the microarray technique,

3) confirmation of the expression of gene candidates using semi quantitative RT-PCR.

Four cell lines were initially studied with the Alamarblue assay in order to detect a cytotoxic effect of PT. The alamarblue assay did not show any killing on the cells at all the concentrations tested (from 0 to 10 μg/ml). This lack of killing effect was not

expected and aggregation of the cells observed by morphological study did suggest that the toxin was having some effect. Therefore, another widely used cytotoxic assay, the MTT assay, was preferred, and did reveal a cytotoxic effect of PT on cell lines. The reason for the discrepancy between the two cytotoxicity assays was not clear and should perhaps be investigated further.

From the four cell lines initially studied, Jurkat, SHSY5Y, NL20 and HUVEC cells, two were chosen to carry out further experiments. Indeed, it would be difficult to perform a comparative study on microarray experiments on four cell lines treated with two different toxin preparations (PT and dPT toxoid). The NL20 cell line (human bronchial epithelial cells) was chosen because bronchial epithelial cells could give valuable information cell responses of located cells at the initial site of infection of *B. pertussis* and also it could give valuable information on the direct cytotoxic effect of PT at the level of gene expression. The second cell type chosen was the HUVEC primary cells (human umbilical vein endothelial cells) because primary cells are sampled directly from humans and their responses are supposed to reflect closely human body reactions. Also HUVEC cells were chosen because they could give valuable information of the role of PT in the induction of encephalopathy, on endothelial cell migration and endothelial cell permeability.

In addition to selection of cell type, toxin preparations had to be chosen too. From the morphological study, cell aggregates were observed after PT treatment. In this study, toxin and corresponding buffer preparations were investigated and it was observed that aggregate formation could be due to the buffer used for the toxin preparation. Indeed some concentrations of toxin in the stock solution were low (e.g. 10 µg/ml), therefore the stock solution of the toxin, in some cases, had to be used neat which resulted in a very high concentration of salt in the cell medium and this presumably

was responsible for inducing the aggregation of the cells. Consequently, PT preparation 2133 at a stock solution concentration of 85 µg/ml was chosen for further experiments (MTT, Microarray, RT-PCR and cell migration). This was the only preparation without any aggregation effect due to the corresponding buffer.

MTT cytotoxicity experiments showed that PT induced cell death in HUVEC and NL20 cells at a concentration of 1.25 µg/ml (10% cell death) with a maximum effect observed at 10 µg/ml (30% cell death). Bruckener *et al.*, (2003) used morphological observations on brain capillary endothelial cells in an *in vitro* system and reported that PT at a concentration up to 1 µg/ml for several days did not result in obvious detrimental or toxic effects. However, concentrations of PT exceeding 2 µg/ml were reported to be lethal for the cells but the percentage of killing was not specified in the report. Our findings also suggested that PT at the concentration of 2.5 µg/ml was cytotoxic, causing approximately 20% of cell death. An initial goal of this study was to try to establish a concentration of active PT that would not greatly affect cell viability but, hopefully, would cause maximal changes, e.g. in gene expression, that could be detected by *in vitro* techniques such as microarray analysis. However, it was difficulty to achieve this because a relatively high concentration of PT was needed to reach the sensitivity required by the *in vitro* system used in the present study. It is also possible that cultured cells *in vitro* were not at an optimal condition for PT receptor binding and thus the cells would have a much reduced sensitivity to the toxic effects of PT. A receptor for PT on all cell types has not yet been identified. The first hypothesis is based on the report that PT recognizes sialoglycoproteins on Jurkat cells (Armstrong *et al.*, 1994), pancreatic b cells (el Baya *et al.*, 1995) and Chinese ovary cells (Brennan *et al.*, 1988). In addition, PT is able to bind glycolipids harbouring terminal sialic acids (Hausman & Burns, 1993). It is possible that HUVEC

and NL20 cells have only a limited amount of sialoglycoproteins on their surface or that the glycoproteins structure could have been disturbed under the *in vitro* condition. Trypsin is an enzyme used in cell culture to detach the cells from the growing surface. The cells are treated with this enzyme for only a few minutes but such a treatment can also lead to deterioration of cell surface receptors when incubated for too long. Trypsin cleaves peptides on the C-terminal side of lysine and arginine amino acid residue. The enzyme could have indirectly damaged the structure of the glycoprotein on the target cell and therefore affected the binding of PT on the cell surface receptor.

Another possible explanation for the low sensitivity of the cells *in vitro* to PT may be related to the property of PT binding to the complement receptor 3 (CD11b/CD18). Complement receptor 3 is known to be the binding site of the ACT toxin (Guermonprez *et al.*, 2001) and FHA haemagglutinin (Relman *et al.*, 1990). Studies carried out on macrophage cells (U937) indicated that PT also bound to CD11b/CD18 receptor (van't Wout *et al.*, 1992; Wong *et al.*, 1996; Wong & Luk, 1997). The distribution of complement receptors on the surface of HUVEC cells has also been investigated, and it was found that the cells expressed complement receptor 1 and 4 but not complement receptor 3 (Langeggen *et al.*, 2002). No study has yet shown that NL20 cells have the complement receptor 3 on their cell surface. It may be that the cells examined in the present study do not have the receptor so far reported for PT.

The present study showed that a detoxified pertussis toxin preparation, which has a cross-linked B oligomer, had no cytotoxic effects on any of the cells at any concentration tested. It appears therefore that both intact B oligomer and A subunit are required to cause morphological modification of the cells or cell death.

In this study, microarray technology was used to investigate the gene expression profiles of NL20 cells (human bronchial epithelial cells) after PT treatment in order to identify gene markers of PT toxicity on the cell type located at the initial site of infection. In addition, gene expression of endothelial cells (HUVEC) was also investigated in order to identify gene markers of pertussis toxicity on endothelial cells. Based on the results of the MTT cytotoxicity study, cells were treated with PT at 2.5 µg/ml for microarray experiments. It has been previously reported that cells treated with a cytotoxic agent for 4 h would respond to the stress by a burst of gene expression (Regnstrom *et al.*, 2003). During these 4 h the number of transcribed genes is high, which may cause the expression profile to be very difficult to analyse. In addition, based on the MTT cytotoxicity assay, 20% killing was observed after 30 min of incubation of the cells in presence of the toxin. This killing, observed after a short time of incubation, could perhaps have been an artefact caused by disturbing the environment of the cells. Also, significant morphological changes were observed from 6 h of incubation. Therefore, according to 1) the report of instability of gene expression from 0 to 4 h of incubation by Regnstrom *et al.* (2003), and 2) our observation of morphological changes from 6 h (section 3.1.1), an incubation time of 6 h was chosen for treatment of the cells in microarray experiments.

In this study, it was shown that the number of differentially-expressed genes could vary greatly between experiments, which indicates a limitation of the microarray technique (van der Spek *et al.*, 2003). The robustness of the internal control was checked but further statistical analysis on the reproducibility between experiments could have been done. A large number of microarray protocols are available and several different softwares can be used to achieve microarray analysis. Therefore it

is very important to spend time on the design of the experiment. In this present study the protocol followed was based on studies already published (Zao *et al.*, 2005). The protocol followed seems suitable for the questions that we asked but further care on quality controls should have been taken. Great care must be taken from the very first steps of the investigation, such as cell treatment. A very large number of cells are required to obtain the amount of RNA needed to run a microarray experiment. It is therefore possible that, in manipulating large number of cells, variations between experiments on the basic cell culture protocols (cell counting, time outside incubator, toxin preparation batches etc...), were made leading to consequent differences in the gene expression profiles. At the RNA extraction level, standardised protocols were followed and quality controls were made by electrophoresis. However, RNA denaturation could have occurred between the time of the quality control and the time of experiment. Regarding the microarray protocol itself, microarray is a succession of very sensitive steps and very low technical variations in the synthesis of the radioactive cDNA, batch of radioactive ^{33}P, the state (first, second or third use) of the membrane coated with oligonucleotides, hybridisation step or even rinsing step can be a source of variation. To counteract the poor reproducibility, a microarray experiment should ideally be composed of three biological replicates and each biological replicate should be composed of three technical replicates. But the amount of data produced this way and the cost of the experiment involved led us to perform three biological replicates each with only one technical replicate.

For further experiments, in addition to taking care in every step prior to microarray analysis, microarray quality controls should be added as explained below. There are three levels at which quality control may be warranted: the probe level, the gene level and the array level. Poor probe quality means poor quality of one particular gene

expression measurement on one particular array. It is recommended that all of the following criteria should be considered, and to eliminate or impute any spot that fails any of them: visual inspection of the image file, spot size, weak signal, large relative background intensity. Poor gene quality means poor quality of the expression measurement for a single gene across all arrays. The tests of poor probe quality consist of checking the poor hybridization and printing, doing probe quality control based on duplicate spots and eliminating genes with low variance. Poor array quality means poor quality of all spots on one particular membrane. Four indicators are available to assess an array quality and, if the array does not pass on these indicators, the array must be redone rather than risk polluting the good data available on the other arrays. The first indicator is the number of spots on the array excluded due to poor quality. This number must not be more than 30%. The second indicator of overall array quality is the ratio of the average of the foreground intensities of the spots on the array, and the average of the background intensities of the spots on the array. This ratio must have a relatively large value. The third indicator is a very low variance of intensities on the control and treatment membranes. The last indicator of array quality is the number of saturated pixels (e.g. if more than 2% of the spots on the array have more than half of their pixels saturated). Regarding the overall microarray analysis, further experiments should strictly follow these five steps: 1) Normalisation 2) Filter 3) Check for robustness 4) Combine the results and 5) Statistical analysis.

Keeping in mind the limitation exposed, we think, however that results produced in this study could give valuable indicators of PT toxicity. To our knowledge, no study has reported using microarray analysis to investigate the role of PT on HUVEC primary cells. One study using a PCR approach showed that pre-treatment of

HUVEC with PT up-regulated mRNA levels and surface expression of ICAM-1 induced by TNF-α (Bernot *et al.*, 2005). Our PCR results (expression level ratio of TNF-α+PT treatment/TNF-α treatment = 5) with HUVEC cells treated with PT and TNF-α were in agreement with these previous findings. It would be interesting in the future to perform microarray analysis of PT+TNF-α treated endothelial cells and TNF-α treated endothelial cells and to compare the gene expression profiles. If the up-regulation of the ICAM 1 gene, specifically by PT+TNF-α treatment, could be confirmed by microarray analysis or real time RT-PCR, the ICAM 1 gene may serve as a gene marker of PT toxicity.

Some genes, i.e. Galectin 3, SAR 1, CHRNA and DPH2L1, identified from the HUVEC microarrays as possibly up-regulated, were further analysed using semi-quantitative RT-PCR. However, positive signals for those genes were obtained in only one RNA sample. The expression ratios observed in the microarray experiments could not be clearly confirmed. The RT-PCR experiment results suggested that ICAM 1 could be down-regulated (expression level of 0.7) by PT + IL1 β treatments. Unfortunately, the experiment was not repeated and the down-regulation of the gene observed in Fig. 16b might have been caused by a lower amount of RNA in the reaction with the RNA samples related to PT + IL1 β treatment compared with the other samples, and not by any direct effect of the treatment on gene expression. The conditions for RT-PCR should be further optimised and the use of a sensitive real-time PCR could be considered.

Similarly, there have been no microarray studies reported on NL20 cells. Nevertheless, the role of PT on epithelial cells has been investigated on BEAS-2B cells (Belcher *et al.*, 2000), a bronchial epithelial cell line which is comparable to NL20 cells. Belcher *et al.* concluded that the quantity and quality of respiratory

secretions were altered by the ADP-ribosyl transferase activity of PT. However, none of the 33 differentially-expressed genes identified by Belcher *et al* was found to be altered in the present microarray study. The CREb1 gene that was found to be up-regulated 37 times compared to the control in their study was included in the present RT-PCR experiment. A signal for this gene was detected in only two experiments and the ratios of the expression levels were 1.79 and 0.9 for HUVEC and NL20 cells, respectively. The variability of the results between Belcher *et al* and our study may be explained as follows: a) different cell types b) the origin and activities of the toxins used c) the concentration and the time of incubation of the toxins and d) the different microarray techniques. The low number of genes up-regulated after PT treatment was noted both by Belcher *et al* and in our study which may allow speculation that, *in vivo*, PT activity might involve interaction with other *B. pertussis* virulence factors (i.e. LPS, FHA).

In the present study on NL20 cells, PT appeared to up-regulate several genes: TNF-α, leucine zipper down-regulated in cancer 1 and the gene encoding the B-cell receptor-associated protein, associated with apoptosis. ACT and FHA, two of the virulence factors of *B. pertussis* have been reported to induce apoptosis of macrophages (Khelef & Guiso, 1995; Abramson *et al.*, 2001), but PT has not been reported to have a direct involvement in apoptosis. PT has been implicated in up-regulation of TNF-α in peripheral blood cells (Boldrick *et al.*, 2002), whereas no evidence of *B. pertussis*-induced apoptosis on BEAS-2b cells was found (Belcher *et al.*, 2000). Therefore TNF-α upregulation by PT may play a minor role in the induction of apoptosis or act as a proinflammatory cytokine, to enhance the cell-mediated immune responses. This hypothesis could be supported by up-regulation of galectin 3, small inducible cytokine subfamily 20, Thy-1 cell surface antigen and CD63 genes

that, according to the literature, are related to induction of immunological responses and were up-regulated with PT treatment in the present study.

The present data also indicate a possible function of PT in altering endothelial permeability (by the finding that genes for platelet-derived growth factor, vascular endothelial growth factor c were up-regualted). Few of the genes up-regulated by PT treatment (glial fibrillary acidic protein, chlorine channel 3, cholinergic receptor) are known to be linked to the brain area and the function of those genes could have an indirect relation with brain damage though to be caused by PT or with a direct effect on vascular permeability leading eventually to brain encephalopathy. Some of these genes involved in endothelial permeability were also up-regulated in NL20 cells. Although it has been reported that PT does not affect the epithelial barrier, PT has been known to modify the morphology of endothelial cells (Bruckener *et al.*, 2003). In addition, although our findings need further investigation, results suggest that PT could induce endothelial cell migration. If PT could modify angiogenesis at the brain level, it could eventually, in this way, be involved in encephalopathy. In previous studies, it had been speculated that *B. pertussis* might increase cerebral vascular permeability (Amiel, 1976). In addition, induction of experimental autoimmune encephalomyelitis (EAE) by PT has been suggested to be due to an increase in vascular permeability (Ben Nun *et al.*, 1997), possibly resulting in histamine-sensitisation (Yong *et al.*, 1993). Therefore, it could be that PT induces effect on the endothelial cells leading to the permeabilization of the vascular vein tissues.

The potential of a permeability assay as an *in vitro* replacement for the HIST test should be explored. As PT induces the migration of the endothelial cells, it could increase the permeability of an endothelial monolayer. *In vitro* assays developed to investigate the permeabilization of endothelial monolayers are well established. One

such assay consists of the growth of endothelial cells to form a monolayer and, after treatment (eg. with PT), the monolayer resistance is assessed. This technique is called TEER for trans-endothelial electrical resistance. The second technique available consists of the growth of endothelial cells on an insert until confluence of the cells is reached. And then, after treatment, the intercellular permeability is assessed by the compartmental exchange of Dextran-FITC. The signal is recorded on 24-well plates by fluorescent spectrophotometry. This test could be easily standardised, fairly rapid is (3 to 4 days) and requires only standard laboratory equipment. In order to develop a permeabilization assay as a control test for pertussis vaccines, a few points would need to be investigated further: a) the dose response to PT and to different vaccine preparation b) the effect of different components of the vaccine (LPS, FHA, $AlPO_4$ etc...) on the permeabilization of the cells c) reproducibility of the assay and d) validation of the assay by international study.

Another potential function of PT is to induce hypoglycaemia by enhancing secretion of insulin by pancreatic islets (el Baya *et al.*, 1995). Interestingly, the present results from the microarray study could suggest that PT-treated cells might counter balance the effect by upregulating genes that increase blood glucose levels (adenylate cyclase-activating polypeptide receptor type 1 and insulin growth factor binding protein 3).

In conclusion, this study has shown that PT induces cell death only at high concentrations. At lower concentrations, PT induces migration of endothelial cells and may consequently play a role in angiogenesis. This study also provides valuable findings for the further investigation of gene regulation in cells after PT treatment. The analysis of the gene expression profiles altered by PT treatment may generate

gene candidates as potential markers for developing an *in vitro* toxicity test for PT. The results suggest a potential function of PT in the permeabilization of endothelial cells monolayers. Therefore, a permeabilization assay as a candidate replacement for the HIST test should be explored.

5. REFERENCES

Abeysinghe, H. R., Cao, Q., Xu, J., Pollock, S., Veyberman, Y., Guckert, N. L., Keng, P., & Wang, N. (2003). THY1 expression is associated with tumor suppression of human ovarian cancer. *Cancer Genet.Cytogenet.* **143**, 125-132.

Abramson, T., Kedem, H., & Relman, D. A. (2001). Proinflammatory and proapoptotic activities associated with *Bordetella pertussis* filamentous hemagglutinin. *Infect.Immun.* **69**, 2650-2658.

Ad hoc group for the study of pertussis vaccines. Placebo-controlled trial of two acellular pertussis vaccines in Sweden-protective efficacy and adverse events. *Lancet* 1, 955-960 (1988).

Adamson, P., Wilbourn, B., Etienne-Manneville, S., Calder, V., Beraud, E., Milligan, G., Couraud, P. O., & Greenwood, J. (2002). Lymphocyte trafficking through the blood-brain barrier is dependent on endothelial cell heterotrimeric G-protein signaling. *FASEB J.* **16**, 1185-1194.

Akioka, H., Forsberg, N. E., Ishida, N., Okumura, K., Nogami, M., Taguchi, H., Noda, C., & Tanaka, K. (1995). Isolation and characterization of the HC8 subunit gene of the human proteasome. *Biochem. Biophys. Res. Commun.* **207**, 318-323.

Akoulitchev, S., Chuikov, S., & Reinberg, D. (2000). TFIIH is negatively regulated by cdk8-containing mediator complexes. *Nature* **407**, 102-106.

Albig, W. & Doenecke, D. (1997). The human histone gene cluster at the D6S105 locus. *Hum. Genet.* **101**, 284-294.

Allavena, P., Bianchi, G., Zhou, D., van Damme, J., Jilek, P., Sozzani, S., & Mantovani, A. (1994). Induction of natural killer cell migration by monocyte chemotactic protein-1, -2 and -3. *Eur.J.Immunol.* **24**, 3233-3236.

Amiel, S. A. (1976). The effects of *Bordetella pertussis* vaccine on cerebral vascular permeability. *Br.J.Exp.Pathol.* **57**, 653-662.

Anand, R. & Lindstrom, J. (1992). Chromosomal localization of seven neuronal nicotinic acetylcholine receptor subunit genes in humans. *Genomics* **13**, 962-967.

Antoine, R., Alonso, S., Raze, D., Coutte, L., Lesjean, S., Willery, E., Locht, C., & Jacob-Dubuisson, F. (2000). New virulence-activated and virulence-repressed genes identified by systematic gene inactivation and generation of transcriptional fusions in Bordetella pertussis. *J.Bacteriol.* **182**, 5902-5905.

Arciniega, J. L., Corbel, M., Dellepiane, N., Dobbelaer, R., Griffiths, E., Heron, I., Ivanoff, B., Kreeftenberg, H., Mastrantonio, P., Meade, B., Milstein, J., Robertson, S., Robinson, A., Sato, H., Sato, Y., Schwanig, M., & Tiru, M. (1998). WHO guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines. *Biologicals* **26**, 195-204.

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

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

Ausiello, C. M., Fedele, G., Urbani, F., Lande, R., Di Carlo, B., & Cassone, A. (2002). Native and genetically inactivated pertussis toxins induce human dendritic cell maturation and synergize with lipopolysaccharide in promoting T helper type 1 responses. *J.Infect.Dis.* **186**, 351-360.

Baker, S. M., Masi, A., Liu, D. F., Novitsky, B. K., & Deich, R. A. (1995). Pertussis toxin export genes are regulated by the ptx promoter and may be required for efficient translation of ptx mRNA in Bordetella pertussis. *Infect.Immun.* **63**, 3920-3926.

Baker, T. K., Carfagna, M. A., Gao, H., Dow, E. R., Li, Q., Searfoss, G. H., & Ryan, T. P. (2001). Temporal gene expression analysis of monolayer cultured rat hepatocytes. *Chem.Res.Toxicol.* **14**, 1218-1231.

Bargatze, R. F. & Butcher, E. C. (1993). Rapid G protein-regulated activation event involved in lymphocyte binding to high endothelial venules. *J.Exp.Med.* **178**, 367-372.

Barlat, I., Maurier, F., Duchesne, M., Guitard, E., Tocque, B., & Schweighoffer, F. (1997). A role for Sam68 in cell cycle progression antagonized by a spliced variant within the KH domain. *J.Biol.Chem.* **272**, 3129-3132.

Barnes, M. G. & Weiss, A. A. (2001). BrkA protein of Bordetella pertussis inhibits the classical pathway of complement after C1 deposition. *Infect.Immun.* **69**, 3067-3072.

Bassinet, L., Gueirard, P., Maitre, B., Housset, B., Gounon, P., & Guiso, N. (2000). Role of adhesins and toxins in invasion of human tracheal epithelial cells by Bordetella pertussis. *Infect.Immun.* **68**, 1934-1941.

Belcher, C. E., Drenkow, J., Kehoe, B., Gingeras, T. R., McNamara, N., Lemjabbar, H., Basbaum, C., & Relman, D. A. (2000). The transcriptional responses of respiratory epithelial cells to Bordetella pertussis reveal host defensive and pathogen counter-defensive strategies. *Proc.Natl.Acad.Sci.U.S.A* **97**, 13847-13852.

Bellalou, J., Sakamoto, H., Ladant, D., Geoffroy, C., & Ullmann, A. (1990). Deletions affecting hemolytic and toxin activities of Bordetella pertussis adenylate cyclase. *Infect.Immun.* **58**, 3242-3247.

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. *Proc.Assoc.Am.Physicians* **109**, 120-125.

Benes, V. & Muckenthaler, M. (2003). Standardization of protocols in cDNA microarray analysis. *Trends Biochem.Sci.* **28**, 244-249.

Berditchevski, F. (2001). Complexes of tetraspanins with integrins: more than meets the eye. *J.Cell Sci.* **114**, 4143-4151.

Bernot, D., Peiretti, F., Canault, M., Juhan-Vague, I., & Nalbone, G. (2005). Upregulation of TNF-alpha-induced ICAM-1 surface expression by adenylate cyclase-dependent pathway in human endothelial cells. *J.Cell Physiol* **202**, 434-441.

Boldrick, J. C., Alizadeh, A. A., Diehn, M., Dudoit, S., Liu, C. L., Belcher, C. E., Botstein, D., Staudt, L. M., Brown, P. O., & Relman, D. A. (2002). Stereotyped and specific gene expression programs in human innate immune responses to bacteria. *Proc.Natl.Acad.Sci.U.S.A* **99**, 972-977.

Bolger, G. B., Stamberg, J., Kirsch, I. R., Hollis, G. F., Schwarz, D. F., & Thomas, G. H. (1985). Chromosome translocation t(14;22) and oncogene (c-sis) variant in a pedigree with familial meningioma. *N.Engl.J.Med.* **312**, 564-567.

Bolgiano, B., Crane, D. T., Xing, D., Williams, L., Jones, C., & Corbel, M. J. (1999). Physico-chemical analysis of *Bordetella pertussis* antigens. *Biologicals* **27**, 155-162.

Boshoff, H. I., Myers, T. G., Copp, B. R., McNeil, M. R., Wilson, M. A., & Barry, C. E. (2004). The transcriptional responses of M. tuberculosis to inhibitors of metabolism: Novel insights into drug mechanisms of action. *J.Biol.Chem.***17**; 279 (38) 40174-84.

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. *J.Biol.Chem.* **263**, 4895-4899.

Brenner, M., Johnson, A. B., Boespflug-Tanguy, O., Rodriguez, D., Goldman, J. E., & Messing, A. (2001). Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander disease. *Nat.Genet.* **27**, 117-120.

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. *Infect.Immun.* **65**, 1114-1118.

Brown, K. L., Birkenhead, D., Lai, J. C., Li, L., Li, R., & Johnson, P. (2005). Regulation of hyaluronan binding by F-actin and colocalization of CD44 and phosphorylated ezrin/radixin/moesin (ERM) proteins in myeloid cells. *Exp.Cell Res.* **303**, 400-414.

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. *J.Cell Sci.* **116**, 1837-1846.

Burgoyne, R. D., Cambray-Deakin, M. A., Lewis, S. A., Sarkar, S., & Cowan, N. J. (1988). Differential distribution of beta-tubulin isotypes in cerebellum. *EMBO J.* **7**, 2311-2319.

Burns, D. L. (1999). Biochemistry of type IV secretion. *Curr.Opin.Microbiol.* **2**, 25-29.

Calvo, D., Dopazo, J., & Vega, M. A. (1995). The CD36, CLA-1 (CD36L1), and LIMPII (CD36L2) gene family: cellular distribution, chromosomal location, and genetic evolution. *Genomics* **25**, 100-106.

Carbonetti, N. H., Artamonova, G. V., Mays, R. M., & Worthington, Z. E. (2003). Pertussis toxin plays an early role in respiratory tract colonization by Bordetella pertussis. *Infect.Immun.* **71**, 6358-6366.

Caro, J.J, Getsios, D., El-Hadi, W., Payne, K; O'Brien, J.A. Pertussis immunization of adolescents in the Unites States: an economic evaluation. (2005). Pediatr. *Infect. Dis. J.* May; **24** (5 Suppl):S75-82.

Chaby R, Caroff M. Lipopolysaccharides of *Bordetella pertussis* endotoxin. *In*: Pathogenesis and immunity in pertussis. Wardlaw AC, Parton R (Eds). John Wiley and Sons, UK, 247-271 (1988).

Chen, Y. T., Scanlan, M. J., Sahin, U., Tureci, O., Gure, A. O., Tsang, S., Williamson, B., Stockert, E., Pfreundschuh, M., & Old, L. J. (1997). A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc.Natl.Acad.Sci.U.S.A* **94**, 1914-1918.

Clark, C. G. & Armstrong, G. D. (1990). Lymphocyte receptors for pertussis toxin. *Infect.Immun.* **58**, 3840-3846.

Cohen H (1969). Relation between toxicity tests in mice and reactions in children using four lots of quadruple vaccine (DTP-polio). *Sym.series immunobiol.stand.* **10**, 53-62.

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.

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

Cowell, J. L., Hewlett, E. L., & Manclark, C. R. (1979). Intracellular localization of the dermonecrotic toxin of *Bordetella pertussis*. *Infect.Immun.* **25**, 896-901.

Cunningham, M. J., Liang, S., Fuhrman, S., Seilhamer, J. J., & Somogyi, R. (2000). Gene expression microarray data analysis for toxicology profiling. *Ann.N.Y.Acad.Sci.* **919**, 52-67.

Cunningham, S. A., Rodriguez, J. M., Arrate, M. P., Tran, T. M., & Brock, T. A. (2002). JAM2 interacts with alpha4beta1. Facilitation by JAM3. *J.Biol.Chem.* **277**, 27589-27592.

de Longueville, F., Atienzar, F. A., Marcq, L., Dufrane, S., Evrard, S., Wouters, L., Leroux, F., Bertholet, V., Gerin, B., Whomsley, R., Arnould, T., Remacle, J., & Canning, M. (2003). Use of a low-density microarray for studying gene expression patterns induced by hepatotoxicants on primary cultures of rat hepatocytes. *Toxicol.Sci.* **75**, 378-392.

DeLong, L. J., Bonamy, G. M., Fink, E. N., & Allison, L. A. (2004). Nuclear export of the oncoprotein v-ErbA is mediated by acquisition of a viral nuclear export sequence. *J.Biol.Chem.* **279**, 15356-15367.

de Melker, H.E, Conyn-van Spaendonck, M.A;, Rumke H.C., van Wijngaarden, J.K, Mooi, F.R, Schellekens, J.F. (1997). Pertussis in The Netherlands: an outbreak despite high levels of immunization with whole -cell vaccine. *Emerg. Infect. Dis.* Apr-Jun **3**(2),175-8.

Dhiman, N., Bonilla, R., O'Kane, D. J., & Poland, G. A. (2001). Gene expression microarrays: a 21st century tool for directed vaccine design. *Vaccine* **20**, 22-30.

Diphtheria, tetanus, pertussis vaccine (adsorbed). In: *European pharmacopoeia*. (4th ed.), 2178-2200. (2002b).

di Tommaso, A., de Magistris, M. T., Bugnoli, M., Marsili, I., Rappuoli, R., & Abrignani, S. (1994). Formaldehyde treatment of proteins can constrain presentation to T cells by limiting antigen processing. *Infect.Immun.* **62**, 1830-1834.

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. *Infect.Immun.* **69**, 4217-4223.

Dono, R., Montuori, N., Rocchi, M., Ponti-Zilli, L., Ciccodicola, A., & Persico, M. G. (1991). Isolation and characterization of the CRIPTO autosomal gene and its X-linked related sequence. *Am.J.Hum.Genet.* **49**, 555-565.

Edwards, K. M., M. D. Decker, and E. A. Mortimer. (1999). Pertussis vaccine. *In* S. A. Plotkin and W. A. Orenstein (Eds.), Vaccines, 3rd ed. W. B. Saunders, Philadelphia, Pa. 293-344.

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.

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

el Baya, A., Linnemann, R., Olleschik-Elbheim, L., & Schmidt, M. A. (1995). Identification of binding proteins for pertussis toxin on pancreatic beta cell-derived insulin-secreting cells. *Microb.Pathog.* **18**, 173-185.

Elliott, K. J., Ellis, S. B., Berckhan, K. J., Urrutia, A., Chavez-Noriega, L. E., Johnson, E. C., Velicelebi, G., & Harpold, M. M. (1996). Comparative structure of human neuronal alpha 2-alpha 7 and beta 2-beta 4 nicotinic acetylcholine receptor subunits and functional expression of the alpha 2, alpha 3, alpha 4, alpha 7, beta 2, and beta 4 subunits. *J.Mol.Neurosci.* **7**, 217-228.

Eskra, L., Mathison, A., & Splitter, G. (2003). Microarray analysis of mRNA levels from RAW264.7 macrophages infected with *Brucella abortus*. *Infect.Immun.* **71**, 1125-1133.

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. *J.Biol.Chem.* **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*. *Infect.Immun.* **68**, 4049-4054.

Finn, T. M. & Amsbaugh, D. F. (1998). Vag8, a *Bordetella pertussis* bvg-regulated protein. *Infect.Immun.* **66**, 3985-3989.

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

Flak, T. A. & Goldman, W. E. (1996). Autotoxicity of nitric oxide in airway disease. *Am.J.Respir.Crit Care Med.* **154**, S202-S206.

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

Forsyth, K.D, Campins-Marti, M., Caro, J., Cherry, J.D, Greenberg, D., Guiso, N., Heininger, U., Schellekens, J., Tan, T., von Konig, C.H., Plotkins, S., Global Pertussis Initiative. New pertussis vaccination strategies beyond infancy: recommendations by the global pertussis initiative. (2004). *Clin. Infect. Dis.* Dec 15;**39**(12):1802-9.

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. *Infect.Immun.* **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. *Infect.Immun.* **60**, 4578-4585.

Gainetdinov, R. R., Bohn, L. M., Sotnikova, T. D., Cyr, M., Laakso, A., Macrae, A. D., Torres, G. E., Kim, K. M., Lefkowitz, R. J., Caron, M. G., & Premont, R. T. (2003). Dopaminergic supersensitivity in G protein-coupled receptor kinase 6-deficient mice. *Neuron* **38**, 291-303.

Galaska A. The immunological basis for immunization. WHO/EPI/GEN/93.11-WHO/EPI/GEN/93.18. (1993). Module 4: Pertussis.

Galindo, C. L., Sha, J., Ribardo, D. A., Fadl, A. A., Pillai, L., & Chopra, A. K. (2003). Identification of *Aeromonas hydrophila* cytotoxic enterotoxin-induced genes in macrophages using microarrays. *J.Biol.Chem.* **278**, 40198-40212.

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. *Infect.Immun.* **65**, 4222-4228.

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

Gierschik, P. (1992). ADP-ribosylation of signal-transducing guanine nucleotide-binding proteins by pertussis toxin. *Curr.Top.Microbiol.Immunol.* **175**, 69-96.

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. *J.Biol.Stand.* **13**, 61-66.

Glaser, P., Sakamoto, H., Bellalou, J., Ullmann, A., & Danchin, A. (1988). Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis*. *EMBO J.* **7**, 3997-4004.

Gray, M. C., Ross, W., Kim, K., & Hewlett, E. L. (1999). Characterization of binding of adenylate cyclase toxin to target cells by flow cytometry. *Infect.Immun.* **67**, 4393-4399.

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.

Guedin, S., Willery, E., Locht, C., & Jacob-Dubuisson, F. (1998). Evidence that a globular conformation is not compatible with FhaC-mediated secretion of the *Bordetella pertussis* filamentous haemagglutinin. *Mol.Microbiol.* **29**, 763-774.

Gueirard, P., Druilhe, A., Pretolani, M., & Guiso, N. (1998). Role of adenylate cyclase-hemolysin in alveolar macrophage apoptosis during *Bordetella pertussis* infection *in vivo*. *Infect.Immun.* **66**, 1718-1725.

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). *J.Exp.Med.* **193**, 1035-1044.

Guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines. In: *WHO technical report series* N° 878, 57-76. (1998).

Guiso, N., Capiau, G., Carletti, G., Poolman, J. and 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., Saxena, S. N., Sharma, S. B., & Ahuja, S. (1988). The effects of purified pertussis components and lipopolysaccharide on the results of the mouse weight gain test. *J.Biol.Stand.* **16**, 321-331.

Hammond, M. E., Lapointe, G. R., Feucht, P. H., Hilt, S., Gallegos, C. A., Gordon, C. A., Giedlin, M. A., Mullenbach, G., & Tekamp-Olson, P. (1995). IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors. *J.Immunol.* **155**, 1428-1433.

Hari, M., Yang, H., Zeng, C., Canizales, M., & Cabral, F. (2003). Expression of class III beta-tubulin reduces microtubule assembly and confers resistance to paclitaxel. *Cell Motil.Cytoskeleton* **56**, 45-56.

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. *Infect.Immun.* **68**, 6720-6728.

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

Hazenbos, W. L., van den Berg, B. M., Geuijen, C. W., Mooi, F. R., & van Furth, R. (1995). Binding of FimD on *Bordetella pertussis* to very late antigen-5 on monocytes activates complement receptor type 3 via protein tyrosine kinases. *J.Immunol.* **155**, 3972-3978.

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

Hershko, A. & Ciechanover, A. (1998). The ubiquitin system. *Annu.Rev.Biochem.* **67**, 425-479.

Hetzel, M., Walcher, D., Grub, M., Bach, H., Hombach, V., & Marx, N. (2003). Inhibition of MMP-9 expression by PPARgamma activators in human bronchial epithelial cells. *Thorax* **58**, 778-783.

Imai, Y., Soda, M., Hatakeyama, S., Akagi, T., Hashikawa, T., Nakayama, K. I., & Takahashi, R. (2002). CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol.Cell* **10**, 55-67.

Imboden, J. B. & Stobo, J. D. (1985). Transmembrane signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. *J.Exp.Med.* **161**, 446-456.

Immunization, vaccines and biologicals: Pertussis vaccine. In: *World health organization website.www.who.com.* (2003).
Ref Type: Electronic Citation

Ishibashi, Y. & Nishikawa, A. (2002). *Bordetella pertussis* infection of human respiratory epithelial cells up-regulates intercellular adhesion molecule-1 expression: role of filamentous hemagglutinin and pertussis toxin. *Microb.Pathog.* **33**, 115-125.
Ishibashi, Y. & Nishikawa, A. (2003). Role of nuclear factor-kappa B in the regulation of intercellular adhesion molecule 1 after infection of human bronchial epithelial cells by *Bordetella pertussis*. *Microb.Pathog.* **35**, 169-177.

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. *J.Biol.Chem.* **274**, 37731-37735.

Janeway C.A and Travers P. (1997). *Immuno biology the immune system in health and disease*, 3rd ed. Current Biology Ltd, London.

Jinquan, T., Frydenberg, J., Mukaida, N., Bonde, J., Larsen, C. G., Matsushima, K., & Thestrup-Pedersen, K. (1995). Recombinant human growth-regulated oncogene-alpha induces T lymphocyte chemotaxis. A process regulated via IL-8 receptors by IFN-gamma, TNF-alpha, IL-4, IL-10, and IL-13. *J.Immunol.* **155**, 5359-5368.

Johnson, J. A., Clark, R. B., Friedman, J., Dixon, R. A., & Strader, C. D. (1990). Identification of a specific domain in the beta-adrenergic receptor required for phorbol ester-induced inhibition of catecholamine-stimulated adenylyl cyclase. *Mol.Pharmacol.* **38**, 289-293.

Jones, B., Jones, E. L., Bonney, S. A., Patel, H. N., Mensenkamp, A. R., Eichenbaum-Voline, S., Rudling, M., Myrdal, U., Annesi, G., Naik, S., Meadows, N., Quattrone, A., Islam, S. A., Naoumova, R. P., Angelin, B., Infante, R., Levy, E., Roy, C. C., Freemont, P. S., Scott, J., & Shoulders, C. C. (2003). Mutations in a Sar1 GTPase of COPII vesicles are associated with lipid absorption disorders. *Nat.Genet.* **34**, 29-31.

Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N., & Alitalo, K. (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J.* **15**, 290-298.

Kamiya, H. & Nii, R. (1988). Overview of currently available Japanese acellular pertussis vaccines and future problems. *Tokai J.Exp.Clin.Med.* **13 Suppl**, 45-49.

Katada, T., Tamura, M., & Ui, M. (1983). The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. *Arch.Biochem.Biophys.* **224**, 290-298.

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

Kendrick PL (1947). Mouse protection tests in the study of pertussis vaccines: a comparative series using the intracerebral route of challenge. *Am.J.Public.Health.* **37**, 803-810.

Khelef, N., Bachelet, C. M., Vargaftig, B. B., & Guiso, N. (1994). Characterization of murine lung inflammation after infection with parental *Bordetella pertussis* and mutants deficient in adhesins or toxins. *Infect.Immun.* **62**, 2893-2900.

Kiefer, M. C., Ioh, R. S., Bauer, D. M., & Zapf, J. (1991). Molecular cloning of a new human insulin-like growth factor binding protein. *Biochem.Biophys.Res.Commun.* **176**, 219-225.

Kosecka, U., Marshall, J. S., Crowe, S. E., Bienenstock, J., & Perdue, M. H. (1994). Pertussis toxin stimulates hypersensitivity and enhances nerve-mediated antigen uptake in rat intestine. *Am.J.Physiol* **267**, G745-G753.

Koths, K., Taylor, E., Halenbeck, R., Casipit, C., & Wang, A. (1993). Cloning and characterization of a human Mac-2-binding protein, a new member of the superfamily defined by the macrophage scavenger receptor cysteine-rich domain. *J.Biol.Chem.* **268**, 14245-14249.

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. *Infect.Immun.* **63**, 3227-3230.

Kurian, K. M., Watson, C. J., & Wyllie, A. H. (1999). DNA chip technology. *J.Pathol.* **187**, 267-271.

Lacobelli, S., Bucci, I., D'Egidio, M., Giuliani, C., Natoli, C., Tinari, N., Rubistein, M., & Schlessinger, J. (1993). Purification and characterization of a 90 kDa protein released from human tumors and tumor cell lines. *FEBS Lett.* **319**, 59-65.

Ladant, D. & Ullmann, A. (1999). Bordatella pertussis adenylate cyclase: a toxin with multiple talents. *Trends Microbiol.* **7**, 172-176.

Langeggen, H., Berge, K. E., Johnson, E., & Hetland, G. (2002). Human umbilical vein endothelial cells express complement receptor 1 (CD35) and complement receptor 4 (CD11c/CD18) in vitro. *Inflammation* **26**, 103-110.

Le Poul, E., Loison, C., Struyf, S., Springael, J. Y., Lannoy, V., Decobecq, M. E., Brezillon, S., Dupriez, V., Vassart, G., van Damme, J., Parmentier, M., & Detheux, M. (2003). Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *J.Biol.Chem.* **278**, 25481-25489.

Lee, J. W., Choi, H. S., Gyuris, J., Brent, R., & Moore, D. D. (1995). Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor. *Mol.Endocrinol.* **9**, 243-254.

Lei, M. G. & Morrison, D. C. (1993). Evidence that lipopolysaccharide and pertussis toxin bind to different domains on the same p73 receptor on murine splenocytes. *Infect.Immun.* **61**, 1359-1364.

Leininger, E., Bowen, S., Renauld-Mongenie, G., Rouse, J. H., Menozzi, F. D., Locht, C., Heron, I., & Brennan, M. J. (1997). Immunodominant domains present on the *Bordetella pertussis* vaccine component filamentous hemagglutinin. *J.Infect.Dis.* **175**, 1423-1431.

Levin, J. & Bang, F. B. (1964). The role of endotoxin in the extracellular coagulation of limulus blood. *Bull.Johns.Hopkins.Hosp.* **115**, 265-274.

Li, H. & Wong, W. S. (2000). Mechanisms of pertussis toxin-induced myelomonocytic cell adhesion: role of CD14 and urokinase receptor. *Immunology* **100**, 502-509.

Livey, I. & Wardlaw, A. C. (1984). Production and properties of *Bordetella pertussis* heat-labile toxin. *J.Med.Microbiol.* **17**, 91-103.

Lloyd, C. M., Delaney, T., Nguyen, T., Tian, J., Martinez, A., Coyle, A. J., & Gutierrez-Ramos, J. C. (2000). CC chemokine receptor CCR3/eotaxin is followed by CCR4/monocyte-derived chemokine in mediating pulmonary T helper lymphocyte type 2 recruitment after serial antigen challenge in vivo. *J.Exp.Med.* **191**, 265-274.

Locht, C., Antoine, R., & Jacob-Dubuisson, F. (2001). *Bordetella pertussis*, molecular pathogenesis under multiple aspects. *Curr.Opin.Microbiol.* **4**, 82-89.

Locht, C., Bertin, P., Menozzi, F. D., & Renauld, G. (1993). The filamentous haemagglutinin, a multifaceted adhesion produced by virulent *Bordetella spp.* *Mol.Microbiol.* **9**, 653-660.

van Loo, IH, Heuvelman, KJ, King A.J, Mooi FR. (2002a). Multilocus sequence typing of *Bordetella pertussis* based on surface protein genes. *J. Clin. Microbiol.* **40** (6): 1994-2001.

van Loo, IH, Mooi FR. (2002b). Changes in the Dutch *Bordetella pertussis* population in the first 20 years after the introduction of whole-cell vaccines. *Microbiology*. **148** (Pt 7):2011-8.

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. *J.Neuroimmunol.* **111**, 68-76.

Luker, K. E., Tyler, A. N., Marshall, G. R., & Goldman, W. E. (1995). Tracheal cytotoxin structural requirements for respiratory epithelial damage in pertussis. *Mol.Microbiol.* **16**, 733-743.

Makhov, A. M., Hannah, J. H., Brennan, M. J., Trus, B. L., Kocsis, E., Conway, J. F., Wingfield, P. T., Simon, M. N., & Steven, A. C. (1994). Filamentous hemagglutinin of *Bordetella pertussis*. A bacterial adhesin formed as a 50-nm monomeric rigid rod based on a 19-residue repeat motif rich in beta strands and turns. *J.Mol.Biol.* **241**, 110-124.

Malcolm, K. C., Arndt, P. G., Manos, E. J., Jones, D. A., & Worthen, G. S. (2003). Microarray analysis of lipopolysaccharide-treated human neutrophils. *Am.J.Physiol Lung Cell Mol.Physiol* **284**, L663-L670.

Masuda, M., Betancourt, L., Matsuzawa, T., Kashimoto, T., Takao, T., Shimonishi, Y., & Horiguchi, Y. (2000). Activation of rho through a cross-link with polyamines catalyzed by Bordetella dermonecrotizing toxin. *EMBO J.* **19**, 521-530.

Masure, H. R. (1993). The adenylate cyclase toxin contributes to the survival of *Bordetella pertussis* within human macrophages. *Microb.Pathog.* **14**, 253-260.

Matsuda, A., Suzuki, Y., Honda, G., Muramatsu, S., Matsuzaki, O., Nagano, Y., Doi, T., Shimotohno, K., Harada, T., Nishida, E., Hayashi, H., & Sugano, S. (2003). Large-scale identification and characterization of human genes that activate NF-kappaB and MAPK signaling pathways. *Oncogene* **22**, 3307-3318.

McGuirk, P. & Mills, K. H. (2000a). A regulatory role for interleukin 4 in differential inflammatory responses in the lung following infection of mice primed with Th1- or Th2-inducing pertussis vaccines. *Infect.Immun.* **68**, 1383-1390.

McGuirk, P. & Mills, K. H. (2000b). Direct anti-inflammatory effect of a bacterial virulence factor: IL-10-dependent suppression of IL-12 production by filamentous hemagglutinin from *Bordetella pertussis*. *Eur.J.Immunol.* **30**, 415-422.

Meagher, L., Mahiouz, D., Sugars, K., Burrows, N., Norris, P., Yarwood, H., Becker-Andre, M., & Haskard, D. O. (1994). Measurement of mRNA for E-selectin, VCAM-1 and ICAM-1 by reverse transcription and the polymerase chain reaction. *J.Immunol.Methods* **175**, 237-246.

Mielcarek, N., Riveau, G., Remoue, F., Antoine, R., Capron, A., & Locht, C. (1998). Homologous and heterologous protection after single intranasal administration of live attenuated recombinant *Bordetella pertussis*. *Nat.Biotechnol.* **16**, 454-457.

Miller, D., N. Madge, J. Diamond J. Wadsworth, and E. Ross. (1993). Pertussis immunisation and serious acute neurological illnesses in children. *Br. Med. J.* **307**:1171-1176.

Miller, L. A. & Butcher, E. C. (1998). Human airway epithelial monolayers promote selective transmigration of memory T cells: a transepithelial model of lymphocyte migration into the airways. *Am.J.Respir.Cell Mol.Biol.* **19**, 892-900.

Mills, K. H. (2001). Immunity to *Bordetella pertussis*. *Microbes.Infect.* **3**, 655-677.

Miyazawa, A., Fujiyoshi, Y., & Unwin, N. (2003). Structure and gating mechanism of the acetylcholine receptor pore. *Nature* **423**, 949-955.

Moggs, J. G., Ashby, J., Tinwell, H., Lim, F. L., Moore, D. J., Kimber, I., & Orphanides, G. (2004). The need to decide if all estrogens are intrinsically similar. *Environ.Health Perspect.* **112**, 1137-1142.

Mooi, F.R, van Loo, IH, King A.J. (2001). Adaptation of *Bordetella pertussis* to vaccination: a cause for its reemergence? *Emerg. Infect. Dis.* **7** (3 suppl), 526-8.

Moii, F.R, van Oirschot, H., Heuvelman, K., van der Heide, H.G.J, Gaastra, W., Willems R.J.L. (1998). Polymorphism in the *Bordetella pertussis* virulence factors P.69/Pertactin and Pertussis toxin in The Netherlands: Temporal trends and evidence for Vaccine-driven evolution. *Infect.Immun.* **66**, N°2. 670-675.

Morbidity and mortality week report. (2005). 54:770.

Morrow, J. A., Collie, I. T., Dunbar, D. R., Walker, G. B., Shahid, M., & Hill, D. R. (1998). Molecular cloning and functional expression of the human glycine transporter GlyT2 and chromosomal localisation of the gene in the human genome. *FEBS Lett.* **439**, 334-340.

Mortimer JR, Edward A . Pertussis. In: *Bacterial infections of humans.* Alfred S.Evans and Philip S.Brachman (Eds). 3rd Edition. Plenum publishing corporation, London, UK. Chap 26, p 529-543 (1998).
Motto, D. G., Ross, S. E., Wu, J., Hendricks-Taylor, L. R., & Koretzky, G. A. (1996). Implication of the GRB2-associated phosphoprotein SLP-76 in T cell receptor-mediated interleukin 2 production. *J.Exp.Med.* **183**, 1937-1943.

Munoz J. Biological activities of pertussigen (pertussis toxin). In: *Pertussis toxin.* Sekura R, Moss J Vaughan M (Eds). Academic press, London, UK, 1-18 (1985).
Ref Type: Serial (Book,Monograph)

Munoz, O., Brignone, C., Grenier-Brossette, N., Bonnefoy, J. Y., & Cousin, J. L. (1998). Binding of anti-CD23 monoclonal antibody to the leucine zipper motif of FcepsilonRII/CD23 on B cell membrane promotes its proteolytic cleavage. Evidence for an effect on the oligomer/monomer equilibrium. *J.Biol.Chem.* **273**, 31795-31800.

Nagasaki, K., Schem, C., von Kaisenberg, C., Biallek, M., Rosel, F., Jonat, W., & Maass, N. (2003). Leucine-zipper protein, LDOC1, inhibits NF-kappaB activation and sensitizes pancreatic cancer cells to apoptosis. *Int.J.Cancer* **105**, 454-458.

Nakagawa, Y., Maeda, H., & Murai, T. (2002). Evaluation of the *in vitro* pyrogen test system based on proinflammatory cytokine release from human monocytes:

comparison with a human whole blood culture test system and with the rabbit pyrogen test. *Clin.Diagn.Lab Immunol.* **9**, 588-597.

Nelson, R. T., Boyd, J., Gladue, R. P., Paradis, T., Thomas, R., Cunningham, A. C., Lira, P., Brissette, W. H., Hayes, L., Hames, L. M., Neote, K. S., & McColl, S. R. (2001). Genomic organization of the CC chemokine mip-3alpha/CCL20/larc/exodus/SCYA20, showing gene structure, splice variants, and chromosome localization. *Genomics* **73**, 28-37.

Nencioni, L., Pizza, M., Bugnoli, M., De Magistris, T., di Tommaso, A., Giovannoni, F., Manetti, R., Marsili, I., Matteucci, G., Nucci, D. . (1990). Characterization of genetically inactivated pertussis toxin mutants: candidates for a new vaccine against whooping cough. *Infect.Immun.* **58**, 1308-1315.

Newton, R. K., Aardema, M., & Aubrecht, J. (2004). The utility of DNA microarrays for characterizing genotoxicity. *Environ.Health Perspect.* **112**, 420-422.

Nielsen, J., Christiansen, J., Lykke-Andersen, J., Johnsen, A. H., Wewer, U. M., & Nielsen, F. C. (1999). A family of insulin-like growth factor II mRNA-binding proteins represses translation in late development. *Mol.Cell Biol.* **19**, 1262-1270.

Nishida, Y., Miki, T., Hisajima, H., & Honjo, T. (1982). Cloning of human immunoglobulin epsilon chain genes: evidence for multiple C epsilon genes. *Proc.Natl.Acad.Sci.U.S.A* **79**, 3833-3837.

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. *J.Cell Physiol* **183**, 91-99.

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.

Ogi, K., Miyamoto, Y., Masuda, Y., Habata, Y., Hosoya, M., Ohtaki, T., Masuo, Y., Onda, H., & Fujino, M. (1993). Molecular cloning and functional expression of a cDNA encoding a human pituitary adenylate cyclase activating polypeptide receptor. *Biochem.Biophys.Res.Commun.* **196**, 1511-1521.

Pan, G., O'Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, R., Ni, J., & Dixit, V. M. (1997). The receptor for the cytotoxic ligand TRAIL. *Science* **276**, 111-113.

Pannese Ennio. In: Neurocytology: Fine structure of Neurons, Nerves Processes, and Neuroglial cells. (1994). p. 165. Thieme, Milano.

Parkhill, J., Sebaihia, M., Preston, A., Murphy, L. D., Thomson, N., Harris, D. E., Holden, M. T., Churcher, C. M., Bentley, S. D., Mungall, K. L., Cerdeno-Tarraga, A. M., Temple, L., James, K., Harris, B., Quail, M. A., Achtman, M., Atkin, R., Baker, S., Basham, D., Bason, N., Cherevach, I., Chillingworth, T., Collins, M., Cronin, A.,

Davis, P., Doggett, J., Feltwell, T., Goble, A., Hamlin, N., Hauser, H., Holroyd, S., Jagels, K., Leather, S., Moule, S., Norberczak, H., O'Neil, S., Ormond, D., Price, C., Rabbinowitsch, E., Rutter, S., Sanders, M., Saunders, D., Seeger, K., Sharp, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Unwin, L., Whitehead, S., Barrell, B. G., & Maskell, D. J. (2003). Comparative analysis of the genome sequences of *Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica*. *Nat.Genet.* **35**, 32-40.

Parry, P., Djabali, M., Bower, M., Khristich, J., Waterman, M., Gibbons, B., Young, B. D., & Evans, G. (1993). Structure and expression of the human trithorax-like gene 1 involved in acute leukemias. *Proc.Natl.Acad.Sci.U.S.A* **90**, 4738-4742.

Pearson, J. D., DeWald, D. B., Mathews, W. R., Mozier, N. M., Zurcher-Neely, H. A., Heinrikson, R. L., Morris, M. A., McCubbin, W. D., McDonald, J. R., Fraser, E. D., & . (1990). Amino acid sequence and characterization of a protein inhibitor of protein kinase C. *J.Biol.Chem.* **265**, 4583-4591.

Perkins FT (1970). The comparison of toxicity of pertussis vaccines in children and mice. *Sym. Series Immunobiol. Stand.* **13**, 41-49.

Phillips, N. J., Zeigler, M. R., & Deaven, L. L. (1996). A cDNA from the ovarian cancer critical region of deletion on chromosome 17p13.3. *Cancer Lett.* **102**, 85-90.

Pizza, M., Covacci, A., Bartoloni, A., Perugini, M., Nencioni, L., de Magistris, M. T., Villa, L., Nucci, D., Manetti, R., Bugnoli, M., & . (1989). Mutants of pertussis toxin suitable for vaccine development. *Science* **246**, 497-500.

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*. *Infect.Immun.* **61**, 2780-2785.

Preston, A. & Maskell, D. J. (2002). A new era of research into *Bordetella pertussis* pathogenesis. *J.Infect.* **44**, 13-16.

Preston, N. W. (1963). Type-specific immunity against whooping-cough. *Br.Med.J.* **5359**, 724-726.

Redhead, K. & Seagroatt, V. (1986). The effects of purified components of *Bordetella pertussis* in the weight gain test for the toxicity testing of pertussis vaccines. *J.Biol.Stand.* **14**, 57-65.

Regnstrom, K., Ragnarsson, E., & Artursson, P. (2003). Gene expression after vaccination of mice with formulations of diphtheria toxoid or tetanus toxoid and different adjuvants: identification of shared and vaccine-specific genes in spleen lymphocytes. *Vaccine* **21**, 2307-2317.

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.

Requirements for diphtheria, tetanus, pertussis and combined vaccines. In: *WHO technical report series* N° 800, 87-149. (1990a).

Requirements for diphtheria, tetanus, pertussis and combined vaccines. In: *WHO technical report series* N°800, 87-149. (1990b).

Reth, M. (1992). Antigen receptors on B lymphocytes. *Annu.Rev.Immunol.* **10**, 97-121.

Robbins, J. B., Pittman, M., Trollfors, B., Lagergard, T. A., Taranger, J., & Schneerson, R. (1993). Primum non nocere: a pharmacologically inert pertussis toxoid alone should be the next pertussis vaccine. *Pediatr.Infect.Dis.J.* **12**, 795-807.

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. *Infect.Immun.* **63**, 2100-2108.

Rogers, J. (2003). The finished genome sequence of *Homo sapiens*. *Cold Spring Harb Symp Quant Biol.* **68**, 1-11.

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. *J.Immunol.* **145**, 678-683.

Rozdzinski, E., Jones, T., Burnette, W. N., Burroughs, M., & Tuomanen, E. (1993). Antiinflammatory effects in experimental meningitis of prokaryotic peptides that mimic selectins. *J.Infect.Dis.* **168**, 1422-1428.

Russell, J. H. & Dobos, C. B. (1983). Accelerated 86Rb+ (K+) release from the cytotoxic T lymphocyte is a physiologic event associated with delivery of the lethal hit. *J.Immunol.* **131**, 1138-1141.

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. *Int.Immunol.* **10**, 651-662.

Sadeghi, M. M., Collinge, M., Pardi, R., & Bender, J. R. (2000). Simvastatin modulates cytokine-mediated endothelial cell adhesion molecule induction: involvement of an inhibitory G protein. *J.Immunol.* **165**, 2712-2718.

Sato H. Pertussis vaccine. In: *Vaccine handbook*. Researcher's Associates, The national institute of health (Eds). Mazuren co. Ltd, Tokyo, Japan. 39-51. (1996).

Ref Type: Serial (Book,Monograph)

Sato, Y. & Arai, H. (1972). Leucocytosis-promoting factor of *Bordetella pertussis*. I. Purification and characterization. *Infect.Immun.* **6**, 899-904.

Sato, Y., Arai, H., & Suzuki, K. (1973). Leukocytosis-promoting factor of *Bordetella pertussis*. II. Biological properties. *Infect.Immun.* **7**, 992-999.

Sato, Y., Arai, H., & Suzuki, K. (1974). Leukocytosis-promoting factor of *Bordetella pertussis*. III. Its identity with protective antigen. *Infect.Immun.* **9**, 801-810.

Sato, Y., Kimura, M., & Fukumi, H. (1984). Development of a pertussis component vaccine in Japan. *Lancet* **1**, 122-126.

Schekman, R. & Orci, L. (1996). Coat proteins and vesicle budding. *Science* **271**, 1526-1533.

Schena, M., Shalon, D., Davis, R. W., & Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**, 467-470.

Schorr, W., Swandulla, D., & Zeilhofer, H. U. (1999). Mechanisms of IL-8-induced Ca2+ signaling in human neutrophil granulocytes. *Eur.J.Immunol.* **29**, 897-904.

Shahin, R. D., Brennan, M. J., Li, Z. M., Meade, B. D., & Manclark, C. R. (1990). Characterization of the protective capacity and immunogenicity of the 69-kD outer membrane protein of *Bordetella pertussis*. *J.Exp.Med.* **171**, 63-73.

Shahin, R. D., Hamel, J., Leef, M. F., & Brodeur, B. R. (1994). Analysis of protective and nonprotective monoclonal antibodies specific for *Bordetella pertussis* lipooligosaccharide. *Infect.Immun.* **62**, 722-725.

Shannon, J. L. & Fernandez, R. C. (1999). The C-terminal domain of the *Bordetella pertussis* autotransporter BrkA forms a pore in lipid bilayer membranes. *J.Bacteriol.* **181**, 5838-5842.

Shirakawa, H. & Yoshida, M. (1992). Structure of a gene coding for human HMG2 protein. *J.Biol.Chem.* **267**, 6641-6645.

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. *Infect.Immun.* **62**, 3108-3114.

Spangrude, G. J., Sacchi, F., Hill, H. R., Van Epps, D. E., & Daynes, R. A. (1985). Inhibition of lymphocyte and neutrophil chemotaxis by pertussis toxin. *J.Immunol.* **135**, 4135-4143.

Stobrawa, S. M., Breiderhoff, T., Takamori, S., Engel, D., Schweizer, M., Zdebik, A. A., Bosl, M. R., Ruether, K., Jahn, H., Draguhn, A., Jahn, R., & Jentsch, T. J. (2001).

Disruption of CIC-3, a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. *Neuron* **29**, 185-196.

Storck, T., von Brevern, M. C., Behrens, C. K., Scheel, J., & Bach, A. (2002). Transcriptomics in predictive toxicology. *Curr.Opin.Drug Discov.Devel.* **5**, 90-97.

Strausberg, R. L., Feingold, E. A., Grouse, L. H., Derge, J. G., Klausner, R. D., Collins, F. S., Wagner, L., Shenmen, C. M., Schuler, G. D., Altschul, S. F., Zeeberg, B., Buetow, K. H., Schaefer, C. F., Bhat, N. K., Hopkins, R. F., Jordan, H., Moore, T., Max, S. I., Wang, J., Hsieh, F., Diatchenko, L., Marusina, K., Farmer, A. A., Rubin, G. M., Hong, L., Stapleton, M., Soares, M. B., Bonaldo, M. F., Casavant, T. L., Scheetz, T. E., Brownstein, M. J., Usdin, T. B., Toshiyuki, S., Carninci, P., Prange, C., Raha, S. S., Loquellano, N. A., Peters, G. J., Abramson, R. D., Mullahy, S. J., Bosak, S. A., McEwan, P. J., McKernan, K. J., Malek, J. A., Gunaratne, P. H., Richards, S., Worley, K. C., Hale, S., Garcia, A. M., Gay, L. J., Hulyk, S. W., Villalon, D. K., Muzny, D. M., Sodergren, E. J., Lu, X., Gibbs, R. A., Fahey, J., Helton, E., Ketteman, M., Madan, A., Rodrigues, S., Sanchez, A., Whiting, M., Madan, A., Young, A. C., Shevchenko, Y., Bouffard, G. G., Blakesley, R. W., Touchman, J. W., Green, E. D., Dickson, M. C., Rodriguez, A. C., Grimwood, J., Schmutz, J., Myers, R. M., Butterfield, Y. S., Krzywinski, M. I., Skalska, U., Smailus, D. E., Schnerch, A., Schein, J. E., Jones, S. J., & Marra, M. A. (2002). Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc. Natl. Acad. Sci. U.S.A* **99**, 16899-16903.

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. *J.Biol.Chem.* **258**, 6756-6761.

Torre, D., Ferrario, G., Bonetta, G., Perversi, L., & Speranza, F. (1996). *In vitro* and *in vivo* induction of nitric oxide by murine macrophages stimulated with *Bordetella pertussis*. *FEMS Immunol.Med.Microbiol.* **13**, 95-99.

Torre, D., Speranza, F., Pugliese, A., Fassina, G., Osculati, A., Perversi, L., Banfi, M. G., & Airoldi, M. (1999). Regulation of inflammatory responses to *Bordetella pertussis* by N(G)-monomethyl-L-arginine in mice intranasally infected. *Mediators.Inflamm.* **8**, 25-29.

Tsien, R. Y., Pozzan, T., & Rink, T. J. (1982). T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. *Nature* **295**, 68-71.

Uhl, M. A. & Miller, J. F. (1995). BvgAS is sufficient for activation of the *Bordetella pertussis* ptx locus in Escherichia coli. *J.Bacteriol.* **177**, 6477-6485.

Ullrich, A., Sures, I., D'Egidio, M., Jallal, B., Powell, T. J., Herbst, R., Dreps, A., Azam, M., Rubinstein, M., Natoli, C., & . (1994). The secreted tumor-associated antigen 90K is a potent immune stimulator. *J.Biol.Chem.* **269**, 18401-18407.

van Amersfoorth, SC., Schouls LM., van der Heide HG. Analysis of *Bordetella pertussis* populations in European countries with different vaccination policies. (2005). *J. Clin. Microbiol.* **43** (6), 2837-43.

van den Berg, B. M., Beekhuizen, H., Willems, R. J., Mooi, F. R., & van Furth, R. (1999). Role of *Bordetella pertussis* virulence factors in adherence to epithelial cell lines derived from the human respiratory tract. *Infect.Immun.* **67**, 1056-1062.

Van der Pouw-Kraan CT, Rensink, H. J., Rappuoli, R., & Aarden, L. A. (1995). Co-stimulation of T cells via CD28 inhibits human IgE production; reversal by pertussis toxin. *Clin.Exp.Immunol.* **99**, 473-478.

van der Spek, P. J., Kremer, A., Murry, L., & Walker, M. G. (2003). Are gene expression microarray analyses reliable? A review of studies of retinoic acid responsive genes. *Genomics Proteomics Bioinformatics.* **1**, 9-14.

van Meijeren, C. E., Vleeming, W., van de, K. T., Gerards, A. L., Hendriksen, C. F., & de Wildt, D. J. (2004a). Pertussis toxin-induced histamine sensitisation: an aspecific phenomenon independent from the nitric oxide system? *Eur.J.Pharmacol.* **493**, 139-150.

van Meijeren, C. E., Vleeming, W., van de, K. T., Manni, J., Kegler, D., Hendriksen, C. F., & de Wildt, D. J. (2004b). *In vivo* pertussis toxin treatment reduces contraction of rat resistance arteries but not that of mouse trachea. *Eur.J.Pharmacol.* **488**, 127-135.

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.

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. *Infect.Immun.* **60**, 3303-3308.

Virok, D., Loboda, A., Kari, L., Nebozhyn, M., Chang, C., Nichols, C., Endresz, V., Gonczol, E., Berencsi, K., Showe, M. K., & Showe, L. C. (2003). Infection of U937 monocytic cells with *Chlamydia pneumoniae* induces extensive changes in host cell gene expression. *J.Infect.Dis.* **188**, 1310-1321.

Vleeming, W., Hendriksen, C. F., van de, K. A., van den Hout, J. W., & de Wildt, D. J. (2000). Mepyramine but not cimetidine or clobenpropit blocks pertussis toxin-induced histamine sensitisation in rats. *Br.J.Pharmacol.* **129**, 1801-1807.

Wang, L., Knudsen, E., Jin, Y., Gessani, S., & Maghazachi, A. A. (2004). Lysophospholipids and chemokines activate distinct signal transduction pathways in T helper 1 and T helper 2 cells. *Cell Signal.* **16**, 991-1000.

Waring, J. F., Ciurlionis, R., Jolly, R. A., Heindel, M., & Ulrich, R. G. (2001). Microarray analysis of hepatotoxins in vitro reveals a correlation between gene expression profiles and mechanisms of toxicity. *Toxicol.Lett.* **120**, 359-368.

Weber, C., Boursaux-Eude, C., Coralie, G., Caro, V., Guiso, N. (2001). Polymorphism of *Bordetella pertussis* isolates circulating for the last 10 years in France, where a single effective whole-cell vaccine has been used for more than 30 years. *J. Clin. Microbiol.* **39** (12), 4396-403.

Weingart, C. L. & Weiss, A. A. (2000). *Bordetella pertussis* virulence factors affect phagocytosis by human neutrophils. *Infect.Immun.* **68**, 1735-1739.

Weiss, A., Imboden, J., Shoback, D., & Stobo, J. (1984). Role of T3 surface molecules in human T-cell activation: T3-dependent activation results in an increase in cytoplasmic free calcium. *Proc.Natl.Acad.Sci.U.S.A* **81**, 4169-4173.

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. *Infect.Immun.* **57**, 3324-3330.

Wong, W. S. & Luk, J. M. (1997). Signaling mechanisms of pertussis toxin-induced myelomonocytic cell adhesion: role of tyrosine phosphorylation. *Biochem.Biophys.Res.Commun.* **236**, 479-482.

Wong, W. S. & Rosoff, P. M. (1996). Pharmacology of pertussis toxin B-oligomer. *Can.J.Physiol Pharmacol.* **74**, 559-564.
Wong, W. S., Simon, D. I., Rosoff, P. M., Rao, N. K., & Chapman, H. A. (1996). Mechanisms of pertussis toxin-induced myelomonocytic cell adhesion: role of Mac-1(CD11b/CD18) and urokinase receptor (CD87). *Immunology* **88**, 90-97.

www.WHO.int/vaccines-diseases/pertussis vaccine.shtml.

Xing, D., Canthaboo, C., Douglas-Bardsley, A., Yuen, C.T, Prior, S., Liu, Y., Corbel, M. (2002a). Development in reduction and replacement of in vivo toxicity and potency tests for Pertussis vaccines. *Advancing science and elimination of the use of laboratory animals for development and control vaccines and hormones.* **111**, 57-68.

Xing, D., Das, R. G., Newland, P., & Corbel, M. (2002b). Comparison of the bioactivity of reference preparations for assaying *Bordetella pertussis* toxin activity in vaccines by the histamine sensitisation and Chinese hamster ovary-cell tests: assessment of validity of expression of activity in terms of protein concentration. *Vaccine* **20**, 3535-3542.

Yamada, Y., Warren, A. J., Dobson, C., Forster, A., Pannell, R., & Rabbitts, T. H. (1998). The T cell leukemia LIM protein Lmo2 is necessary for adult mouse hematopoiesis. *Proc.Natl.Acad.Sci.U.S.A* **95**, 3890-3895.

Yang, Q. S., Xia, F., Gu, S. H., Yuan, H. L., Chen, J. Z., Yang, Q. S., Ying, K., Xie, Y., & Mao, Y. M. (2002). Cloning and expression pattern of a spermatogenesis-related gene, BEX1, mapped to chromosome Xq22. *Biochem.Genet.* **40**, 1-12.

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. *J.Neuroimmunol.* **45**, 47-52.

Zagon, I. S., Verderame, M. F., Allen, S. S., & McLaughlin, P. J. (2000). Cloning, sequencing, chromosomal location, and function of cDNAs encoding an opioid growth factor receptor (OGFr) in humans. *Brain Res.* **856**, 75-83.

Zhang, X. M., Berland, R., & Rosoff, P. M. (1995). Differential regulation of accessory mitogenic signaling receptors by the T cell antigen receptor. *Mol.Immunol.* **32**, 323-332.

Zhang, Y., Guan, D. L., Xia, C. Q., Han, Z. Y., Xu, J. J., Gao, J. Z., & Wu, K. R. (2003). Relationship between the expression levels of CD61, CD63, and PAC-1 on platelet surface in peripheral blood and the transplanted kidney function. *Transplant.Proc.* **35**, 1360-1363.

Zhao Y, Azam S, Thorpe R. (2005). Comparative studies on cellular genes regulation by HIV-1 based vectors: implications for quality control of vector production. *Gene therapy*. **12**, 311-319.

Zhao Y, Xing. Determination of gene expression in murine splenic cells after immunisation with pertussis vaccines. Seventy Int.Symposium Pertussis: genome, pathogenesis and immunity. (2003).

Zidek, Z. (1999). Adenosine - cyclic AMP pathways and cytokine expression. *Eur.Cytokine Netw.* **10**, 319-328.

Zumbihl, R., Dornand, J., Fischer, T., Cabane, S., Rappuoli, R., Bouaboula, M., Casellas, P., & Rouot, B. (1995). IL-1 stimulates a diverging signaling pathway in EL4 6.1 thymoma cells. IL-2 release, but not IL-2 receptor expression, is sensitive to pertussis toxin. *J.Immunol.* **155**, 181-189.

DECLARATION

I declare that I have personally prepared this report and that it has not in whole or in part been submitted for any other degree or qualification. The work described here is my own, carried out personally otherwise stated. All sources of information, including quotations, are acknowledged by means of reference.