# *Bordetella bronchiseptica* dermonecrotic toxin, purification and characterisation

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# Statement

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Toni Elizabeth Adams

# Bordetella bronchiseptica dermonecrotic toxin, purification and characterisation

## Toni Adams

Dermonecrotic toxin (DNT) is produced by all the *Bordetella* species, and DNT from *B. bronchiseptica* is considered to be an important virulence factor in turbinate atrophy of pigs.

Recombinant DNT (rDNT) was purified by sonication, ion-exchange and hydroxylapatite chromatography. Other methods for the purification of wild-type DNT and rDNT, including preparative isoelectric focusing and hydrophobic chromatography, were investigated in detail.

Partially pure preparations of rDNT contained a 145 kDa protein band and were cytotoxic to embryonic bovine lung (EBL) cells. Partially pure rDNT induced the formation of actin stress fibres and focal adhesions in Swiss 3T3 cells. In addition, rDNT stimulated DNA synthesis in quiescent Swiss 3T3 cells but prevented cell proliferation, resulting in binucleated cells. Recombinant DNT has been shown to directly modify the small GTP-binding protein, Rho, (Pullinger, unpublished), which regulates the cell cytoskeleton. Results from this thesis indicate that rDNT causes the assembly of actin stress fibres and focal adhesion possibly by direct activation of the Rho protein.

Partially purified rDNT with a site-directed mutation in a putative nucleotide-binding motif did not induce cytoskeletal rearrangements and did not stimulate DNA synthesis in Swiss 3T3 cells. This suggests that the nucleotide-binding motif is essential for activity.

Two lines of evidence indicate that the toxin is internalised in the endosomal/ lysosomal compartment: i) stimulation of DNA synthesis by transient exposure of Swiss 3T3 cells to rDNT, and ii) blocking of rDNT-induced DNA synthesis with methylamine.

Three monoclonal antibodies (mAbs) were produced against *B. bronchiseptica* DNT. These mAbs recognised rDNT and *B. pertussis* DNT, but none neutralised the cytotoxic activity of DNT on EBL cells.

The partial purification of rDNT and characterisation of its biological effects provide valuable information for further studies of the toxin, including analysis of its enzymatic mode of action and its role in infection. Also, DNT may prove to be a useful tool for analysis of cell responses involving the important signalling molecule, Rho.

# Abbreviations

| | |
|---|---|
| aa | amino acid |
| AC/HA | adenylate cyclase/haemolysin |
| ATP | adenosine triphosphate |
| BCA | bicinchonic acid |
| BSA | bovine serum albumin |
| $^{\circ}C$ | degrees Celcius |
| cAMP | cyclic adenosine 3' 5' monophosphate |
| CAPS | 3-[Cyclohexylamino]-1-propanesulfonic acid |
| Ci | Curie |
| CNF | cytotoxic necrotizing factor |
| cpm | counts per minute |
| DEAE | diethylaminoethyl |
| DNA | deoxyribonucleic acid |
| DNT | dermonecrotic toxin |
| rDNT | recombinant dermonecrotic toxin |
| DMEM | Dulbecco's modified Eagles medium |
| EBL | embryonic bovine lung |
| ELISA | enzyme-linked immunosorbent assay |
| FCS | foetal calf serum |
| FHA | filamentous heamagglutinin |
| g | gram |
| $g$ | gravity ($9.81\ ms^{-2}$) |
| h | hour |
| HCl | hydrochloric acid |
| HIC | hydrophobic interaction chromatography |
| IEF | isoelectric focusing |
| Ig | immunoglobulin |
| IPTG | isopropyl-B-D-thiogalactopyranoside |
| kb | kilobase |
| Da | dalton |
| l | litre |
| LB | Luria-Bertani (medium) |

| | |
|---|---|
| LPS | lipopolysaccharide |
| M | molar |
| mAb | monoclonal antibody |
| min | minute |
| Mol. wt | molecular weight |
| OD | optical density |
| p | plasmid |
| PAGE | polyacrylamide gel electrphoresis |
| PBS | phosphate buffered saline |
| pI | isoelectric point |
| PMT | *Pasteurella multocida* toxin |
| SDS | sodium dodecyl sulphate |
| TCT | tracheal cytotoxin |
| Tris | Tris (hydroymethyl)-methylamine |
| V | volt |
| v/v | volume per volume |
| w/v | weight per volume |
| YT | yeast-tryptone (medium) |

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# Chapter 1
# General Introduction

## 1.1 Bacterial Virulence Factors

Microbial pathogenicity has been defined as "the biochemical mechanisms whereby microorganisms cause disease" (Smith, 1968). Not all pathogens have an equal probability of causing disease. Virulence varies not only between different bacterial species, but also between strains of the same species. Humans and animals have an abundant and varied microbial flora; most of these bacteria do not cause disease, but achieve a balance with the host that ensures the survival, growth, and multiplication of the bacteria and host. However, nonpathogenic microorganisms can occasionally cause disease in compromised individuals and act as opportunistic pathogens. Therefore, the outcome of the interaction between bacteria and host is determined by characteristics that favour the establishment of the bacteria within the host and their ability to damage the host.

Microbial pathogenesis is usually complex and multifactorial. Many factors determine the virulence of bacteria. Firstly, the bacteria must enter and establish themselves within the host. The most common routes of entry are the respiratory, gastrointestinal, and genitourinary tracts. Damaged skin, resulting from cuts or burns is also a frequent site of entry. Once inside the host, the bacteria must adhere to host cells and establish a primary site of infection. Invasion of host cells and tissue leads to damage of cells in the immediate vicinity of the infection. Finally, the production of potent toxins, which may act at remote sites, allow the survival, multiplication, and spread of bacteria within and exit from the host.

In the next section I will give a general overview of bacterial virulence factors, and then discuss in more detail bacterial protein toxins, considering their secretion, internalisation, and modes of action. In the second half of the introduction I will concentrate on species of *Bordetella*, with reference to their virulence factors, especially their toxins. Then I will discuss the role of *Bordetella bronchiseptica* in atrophic rhinitis of pigs, and finally describe the aims of my project.

### 1.1.1 Adherence factors

An important initial step in bacterial infection via mucosal sites is the adhesion of the pathogenic bacteria to the host cells or tissue. This prevents the bacteria from being swept away by mucus and other fluids that bathe the tissue surface. Many

bacteria have several distinct and alternative mechanisms of cell adherence. Frequently, several act in collaboration to ensure adherence to target cells, thereby providing a selective advantage. Fimbriae (pili), produced by many species, enable bacteria to bind to D-mannose residues on eukaryotic cell surfaces (de Graaf and Mooi, 1986). Another type of fimbriae, found in species of *Pseudomonas, Neisseria* and *Vibro*, is that containing N-methylphenylalanine, which allows bacteria to bind to oligosaccharides on eukaryotic cell surfaces (Frost *et al.*, 1978). Fimbriae from group A streptococci composed of lipoteichoic acid, allow binding to fibronectin, a glycoprotein which promotes adherence functions in eukaryotic cells (Beachey and Courtney, 1987). Filamentous haemagglutinin (FHA) of *Bordetella* species exploits common eukaryotic mechanism for cell-cell interactions for adherence to cells (see Section 1.6.1).

### 1.1.2 Invasion

Invasion of host cells is a specialised strategy for survival and multiplication used by a number of bacterial pathogens. Bacteria known to invade cells include obligate intracellular bacteria, such as *Chlamydia* and *Rickettsia* species, and facultative intracellular bacteria, such as *Shigella, Salmonella, Yersinia* and *Listeria* species (Falkow *et al.*, 1992). Invasion of cells allows the bacteria to avoid the host immune system and provides a nutrient rich environment. *Listeria monocytogenes*, the best characterised intracellular pathogen is able to induce its own phagocytosis by non-phagocytic cells (Cossart and Kocks, 1994; Lasa and Cossart, 1996). After being engulfed, the bacteria are trapped in a membrane vacuole which is lysed by bacterial enzymes. The bacteria are released into the cytoplasm where they multiply. In the cytoplasm the bacteria induce actin polymerisation which is the driving force for intracellular movement (Cossart and Kocks, 1994; Lasa and Cossart, 1996). At the plasma membrane, the bacteria cause the formation of long cellular protrusions, each containing a bacterium at the tip. These protrusions are internalised by neighbouring cells and a new cycle of infection begins (Cossart and Kocks, 1994; Lasa and Cossart, 1996). The surface proteins, ActA of *L. monocytogenes* and IcsA of *Shigella flexneri* are involved in actin assembly and bacterial movement (Lasa and Cossart, 1996). No enzymatic function has been identified for ActA. In contrast, IcsA binds ATP and has

ATPase activity (Goldberg *et al.*, 1993). ActA and IcsA do not share any sequence homology, and the exact mechanism of ActA and IcsA induced actin polymerisation is unknown.

### *1.1.3 Extracellular Enzymes*

Many bacteria produce extracellular enzymes which are not intrinsically toxic, but do play an important role in infection. Some of these enzymes are discussed below.

Collagenase: *Clostridium perfringens* produces the proteolytic enzyme collagenase, which degrades collagen, the major protein of fibrous connective tissue. The breakdown of collagen promotes the spread of the bacteria (Matushita *et al.*, 1994).

Hyaluronidase: Many Gram-positive bacteria produce hyaluronidase, which hydrolyses hyaluronic acid, a constituent of the ground substance of connective tissue. The breakdown of hyaluronic acid facilitates bacterial spread through tissues (Berry *et al.*, 1994; Lin *et al.*, 1994).

Coagulase: *Staphylococcus aureus* produces coagulase, an enzyme that clots plasma by a thrombokinase-like action, in the presence of a serum factor, coagulase reacting factor (CRF). Coagulase is responsible for the characteristic fibrin walls that surround staphylococcal lesions, and also causes the deposit of fibrin on the surface of the bacteria, which may protect against phagocytosis (Boden and Flock, 1989).

Streptokinase: *Streptococcus spp.* produce streptokinase which converts plasminogen to plasmin which catalyses the lysis of fibrin and consequently aids the spread of the bacteria through host tissue (Castellino *et al.*, 1979).

IgA protease: IgA protease is produced by *Neisseria spp.*, *Haemophilus influenzae* and *Streptococcus pneumoniae*. IgA protease cleaves immunoglobulin A at a specific proline-threonine or proline-serine bond near the hinge region of the heavy chains of the immunoglobin. This allows the bacteria to inactivate the antibody found on mucosal surfaces (Plaut, 1983).

## 1.1.4 Endotoxins

Bacterial toxins are generally classified into two groups, endotoxins and protein toxins (exotoxins). The main features of the two groups are shown in Table 1.1.

Endotoxins of Gram-negative bacteria are heat-stable, complex lipopolysaccharides (LPS) derived from bacterial cell walls. LPS consists of three regions: polysaccharide side-chains, core polysaccharide, and lipid A. The lipid A region is responsible for toxicity (Schletter *et al.*, 1995; Ulevitch and Tobias, 1995) The pathological effects of endotoxins are similar regardless of their bacterial origin. Endotoxins cause fever, hypotension and shock, impaired perfusion of essential organs, activation of the C3 and complement cascade, intravascular coagulation and death (Ulevitch and Tobias, 1995). LPS binds to CD14, a glycosyl phosphatidylinositol (GPI)-linked membrane protein, found on the surface of monocytes, macrophages and polymorphonuclear leucocytes. Binding of LPS to CD14 is facilitated by a serum factor known as lipopolysaccharide-binding protein. This forms high-affinity complexes with LPS, which are recognised by CD14. Binding of LPS to CD14 stimulates the host cells to produce and release endogenous mediators, such as interleukin-1 (IL-1), IL-6 and tumour necrosis factor alpha (TNFα). The presence of high amounts of LPS leads to the release of these mediators in large quantities, resulting in the described pathological effects (Schletter *et al.*, 1995; Ulevitch and Tobias, 1995).

## 1.1.5 Bacterial protein toxins

Bacterial protein toxins are produced by both Gram-negative and Gram-positive bacteria and nearly 300 bacterial protein toxins have now been identified (Alouf, 1994). Although they are produced in relatively small quantities, bacterial toxins are extremely potent, and the interaction of these prokaryotic proteins with eukaryotic cells results in the host cells being adversely affected or killed. The toxins of *Corynebacterium diphtheriae*, *Clostridium tetani* and *Clostridium botulinum* are amongst the most powerful poisons known. In a mouse, the 50% lethal dose for tetanus or botulinum neurotoxin is between 0.1-1 ng kg$^{-1}$ (Montecucco and Schiavo, 1995), and one molecule of diphtheria toxin fragment A inside a cell results in host cell death (Yamaizmi *et al.*, 1978).

| Protein toxin (exotoxin) | Endotoxin |
|---|---|
| polypeptides | lipopolysaccharride complexes |
| produced by both Gram-positive and Gram-negative bacteria | found only in Gram-negative bacteria |
| generally secreted by living cells | intergral part of the cell wall of Gram-negative bacteria |
| relatively unstable; toxicity lost by heating to about 60°C | Relatively stable; withstand heating to above 60°C for several hours |
| highly antigenic | weakly immunogenic |
| convert to antigenic, nontoxic toxoids by formalin, heat treatment | not converted to toxoids |

Table 1.1 General characteristics of bacterial protein toxins (exotoxins) and endotoxins.

It is assumed that toxin production confers a selective advantage to the bacteria. Toxin damage to the host often allows persistence and multiplication of the bacteria. For example, *Clostridium* toxins kill the surrounding tissue thereby creating a favourable anaerobic environment for optimal bacterial growth and the production of spores (Niemann *et al.*, 1994). Prolonged survival of bacterial cells would be caused by toxins which kill cells of the immune system, such as *B. pertussis* adenylate-cyclase/haemolysin which induces apoptosis in macrophages (Khelef *et al.*, 1993). Bacterial toxins have evolved to target eukaryotic cell components and pathways which are of vital importance. For example, the diphtheria toxin inhibits protein synthesis by ADP-ribosylation of elongation factor 2 (Boquet and Gill, 1991). Many toxins are host- or even cell-specific, depending on specific toxin-cell receptor interactions.

Research into bacterial toxins is not only necessary to understand their role in pathogenesis, but also provides a powerful tool in cell biology research. Bacterial toxins have provided an insight into areas such as: membrane translocation, protein transport and trafficking, neurotransmission, and cell signalling pathways which are disrupted by toxins. For example, research into the molecular mode of action of cholera and pertussis toxins has increased the understanding of the adenylate cyclase cell signalling pathway (Middlebrook and Dorland, 1984), and shiga toxin research has provided information of the retrograde transport of cell surface ligands (Sandvig *et al.*, 1992).

## 1.2 Secretion of bacterial protein toxins

In order for toxins to be effective they must first be secreted across the bacterial outer membrane. Most bacteria have at least two secretory pathways. In Gram negative bacteria the general secretory pathway is a two-step process where proteins cross the inner membrane by the signal peptide-dependant general export pathway (GEP) into the periplasm. Then the protein is secreted across the outer membrane by various mechanisms. This process is dependent on the protein being synthesised with an N-terminal signal sequence, which directs the protein to the cytoplasmic membrane (Wandersman, 1992; Pugsley, 1993). Bacterial toxins known

to contain this signal sequence and to be secreted by this pathway include cholera toxin, *E. coli* heat-labile enterotoxins and aerolysin (Pugsley, 1993).

The second pathway involves secretion of proteins that lack a cleavable hydrophobic signal sequence. Many toxins are known to be secreted by ATP-dependent transporter proteins, known as ABC (ATP binding cassette) transporters (Wandersman, 1992; Kuchle, 1993). The best characterised prokaryotic ABC secretion system is the export machinery for *E. coli* haemolysin A (HlyA). Five genes *hlyA, hlyB, hlyC, hlyD* and *tolC* are required for the synthesis and secretion of HlyA (Mackman *et al.*, 1986; Wandersman and Delepelaire, 1990). After synthesis HlyA is activated by post translational fatty acid acylation by HlyC (Issartel *et al.*, 1991). The HlyB protein, which contains one ATP-binding domain and six possible transmembrane domains, forms a homodimer to create a pore for the toxin to cross both bacterial membranes. The role of HlyD and TolC is unclear, but they possibly act together with HlyB to form the pore.

A new family of proteins has been identified which is involved in the secretion of pertussis toxin from *B. pertussis*. The operon *ptl* (pertussis toxin liberation) was located downstream of the pertussis toxin operon and contains seven genes which are homologous to *Agrobacterium tumefacens virB* genes, which code for proteins involved in the transport of T-DNA across bacterial membranes (Covacci and Rappuoli, 1993; Weiss *et al.*, 1993).

Some bacterial toxins may be dependent on cell lysis for release. *B. bronchiseptica* DNT (Cowell *et al.*, 1979), *Pasteurella multocida* toxin (PMT) (Nakai *et al.*, 1985) and *E. coli* cytotoxic necrotizing factor (CNF) types 1 and 2 (Falbo *et al.*, 1993; Oswald *et al.*, 1994) all have an intracellular location. All these toxins lack the N-terminal signal sequence (Lax *et al.*, 1990; Falbo *et al.*, 1993; Oswald *et al.*, 1994; Pullinger *et al.*, 1996). Both DNT and PMT have been shown to be important in the pathogenesis of atrophic rhinitis in pigs (Chanter *et al.*, 1986; Magyar *et al.*, 1988) which strongly suggests that the toxins must be released from the bacteria during natural infection.

## 1.3 Interaction of bacterial protein toxins with host cells - receptor binding and internalisation

Most toxins that act on intracellular targets exploit the endocytic pathway and enter the cytoplasm of target cells from intracellular compartments, rather than directly through the plasma membrane. Most of these toxins are structurally similar, and consist of two functionally different polypeptides, usually linked by a disulphide bond. The A fragment carries enzyme activity, while the B fragment binds to cell surface receptors. Some of these toxins, such as diphtheria and tetanus toxins, are synthesised as a single polypeptide chain and are proteolytically cleaved to the two chain disulphide linked A-B molecules. Other toxins, such as cholera and pertussis toxins, are synthesised separately and then associated to form the A-B structure (Olsnes et al., 1993). All these toxins share a common mechanism of cell entry, binding via a receptor on the target cell, membrane penetration, and in some cases membrane translocation, and finally, the intracellular modification of target molecule (Olsnes et al., 1993; Montecucco et al., 1994).

Toxins utilise receptors on host cells that were developed for other purposes. Some of the toxin receptors have been identified. *Pseudomonas aeruginosa* exotoxin A binds to and exploits the transmembrane protein receptor for $\alpha_2$ macroglobulin (Kounnas et al., 1992). The diphtheria toxin receptor is a transmembrane protein which is a precursor for heparin-binding epidermal growth factor (Naglich et al., 1992). Once the toxin is bound to the cell surface internalisation occurs by endocytosis either via clathrin-coated pits as has been shown for the diphtheria toxin (Moya et al., 1985) or non-coated pits as has been shown for cholera toxin (Tran et al., 1987).

After endocytosis, the toxin reaches the endosomes or lysosomes, where the toxin penetrates the endosomal membrane and the enzymatic A fragment is released into the cytoplasm. Cell membrane penetration and translocation is best understood for the diphtheria toxin (London, 1992). Exposure of the toxin to low pH induces partial unfolding in the B fragment of the diphtheria toxin, and this exposes hydrophobic regions which are normally hidden. This partial unfolding triggers insertion of the fragment B into the membrane forming cation-selective channels which allows the direct translocation of fragment A across the plasma membrane (Sandvig and Olsnes, 1988). Falnes and Olsnes (1995) suggested a model for the translocation of fragment

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A. The transmembrane domain of the B fragment is inserted into the membrane at low pH and this pulls the N-terminal of the B fragment with the C-terminal part of fragment A, which are linked by the interfragment disulphide bond, into the cytosol. The disulphide bond is reduced and the fragment A is released into the cytoplasm from where it can modify its target and disrupt cell function.

## 1.4 Mechanisms of action of bacterial protein toxins

Bacterial protein toxins have a wide range of mechanisms of action on a wide variety of different eukaryotic targets. Some general patterns have emerged, allowing bacterial toxins to be classified as either intracellular enzymatic toxins, or extracellular acting toxins. The following sections provide a general overview of bacterial toxins, aimed at illustrating the range of producing bacteria, toxins, targets and mechanisms of actions.

### 1.4.1 Extracellular acting toxins

Extracellular acting toxins attack the extracellular surfaces of host cells and tissue and do not require uptake into host cells. These toxins can be divided into two groups; i) toxins that damage host cell membranes through either the insertion of the protein into the membrane, forming discrete transmembrane channels, or direct enzymatic attack on membrane lipids, and ii) toxins that act as superantigens.

### 1.4.1.1 *Staphylococcus aureus* α toxin

The α toxin was the first cytolytic toxin for which pore-forming ability was demonstrated. The α toxin is a soluble monomer that attacks a variety of cells including erythrocytes, polymorphonuclear granulocytes, platelets and endothelial cells (Bhakdi and Tranum-Jensen, 1991). The α toxin binds to an unidentified receptor in the cell membrane. After binding to membranes, the monomer oligomerises to form a non-lytic heptameric pore. The pores allow the leakage of ions and low molecular weight molecules from the cell, causing cell death due to the rapid loss of vital molecules such as ATP and an influx of calcium ions which disrupt cellular functions (Bhakdi and Tranum-Jensen, 1991; Bhakdi *et al.*, 1996). Little is known of how α toxin inserts into the membrane, but studies have shown that insertion is favoured by

acidic pH and that partial C-terminal unfolding is required for channel-formation (Vécsey-Semjén et al., 1996).

## 1.4.1.2 Aerolysin

Aerolysin is a cytotoxin produced by *Aeromonas hydrophila* and the only member of the channel forming toxins for which the three dimensional structure is known (Parker et al., 1994). Aerolysin is a homodimer in solution and the toxin is concentrated on the surface of the target cell by binding to a specific receptor. Proteolytic nicking at the activation site and dissociation of the dimer is required for oligmerisation. This results in the exposure of the hydrophobic regions of the toxin allowing membrane insertion of the oligomer. The channels formed are anion selective and resemble porins in structure (Parker et al., 1996).

## 1.4.1.3 RTX family

The RTX (repeats in toxin) cytolytic toxins all contain a common series of glycine-rich nine amino acids repeats at the C-terminal end of the proteins. The RTX family includes the pore forming toxins from a variety of Gram-negative bacteria including haemolysins from *E. coli*, *Proteus vulgaris* and *Actinobacillus pleuropneumoniae*, leucotoxins from *Pasteurella haemolytica* and *Actinobacillus actinomycetemcomitans*, and the adenylate-cyclase/haemolysin from *Bordetella spp.* (Coote, 1992; Welch et al., 1995). All the toxins generate ion-permeable transmembrane channels in the cytoplasmic membrane of target cells, leading to osmotic lysis. These toxins require the presence of bound calcium ions and post-translational modification in order to show activity. Although the exact structure of the pores formed by RTX toxins is unknown, Ludwig et al. (1993) suggested that two or more *E. coli* haemolysin molecules aggregate prior to pore formation. Comparison of the hydrophobic properties of different RTX toxins indicates a conserved cluster of 10 contiguous amphipathic helixes at the N-terminal which may be involved in channel formation (Menestrina et al., 1994).

### 1.4.1.4 Thiol-activated toxins

Thiol-activated toxins are a large family of homologous cytolysins produced by Gram-positive bacteria. Streptolysin O produced by group A streptococci is the best understood of the thiol-activated toxins (Bhakdi *et al.*, 1996). All these toxins are oxygen-labile and specifically bind cholesterol in the target lipid bilayer. The toxins bind as a monomer to cholesterol and then oligomerise, forming ring and arc structures in the cell membrane (Bhakdi *et al.*, 1985; Morgan *et al.*, 1994). These transmembrane pores cause lysis of the cell, and at sublytic concentrations the toxins impair the function of many cells, especially those from the immune system (Alouf and Geoffrey, 1991).

### 1.4.1.5 Phospholipase C

Phospholipases C have been isolated from a wide variety of Gram-positive and Gram-negative bacteria including *Clostridium perfringens, Listeria monocytogenes* and *Pseudomonas aeruginosa. C. perfringens* α toxin and *L. monocytogenes* PLC B are zinc-metalloenzymes, containing several zinc ions and are reversibly inactivated by EDTA. Phospholipase C enzymatically attacks the phospholipids in the target cell membranes to produce diacylglycerol and inositol triphosphate. All are able to hydrolyse phosphatidylcholine, and other phospholipids, such as sphingomyelin and phosphatidlgycerol. The membrane damage caused by phospholipase C can result in cell lysis, but the more subtle effects of phospholipase C on the metabolism of cells could play an important role in the disease process. At sublytic concentrations, diacylglycerol may accumulate resulting in the activation of the arachidonic acid cascade leading to the production of potent mediators inflammatory responses or the induction of chloride ion secretion. Diacylglycerol can also activate protein kinase C which is known to modulate a wide variety of cell processes (Titball, 1993).

### 1.4.1.6 Superantigens

This group of toxins bind to proteins on the cell surface of the target cell and stimulate transmembrane signals which interfere with cell signalling. They include toxins produced by several different bacteria including *Streptococcus pyogenes, Mycoplasma arthriditis,* and *Staphylococcus aureus* which produces the staphylococcal

enterotoxins and the toxic shock syndrome toxin. These toxins bind to the major histocompatibility complex (MHC) class II and to the $V_\beta$ region of the T cell receptor. The model for toxin binding shows that the toxin cross-links the MHC class II and $V_\beta$ bringing together the T cell receptor and MHC. This stimulates the T cell immune response, resulting in the over production of cytokines causing symptoms of shock, fever, hypotension and dysfunction of organ systems. Some of these toxins can cause autoimmune diseases, such as rheumatic fever, which may be due to stimulation of T cells that are potentially autoreactive (Marrack and Kappler, 1990; Fleischer, 1994).

### 1.4.2 Intracellular acting toxins

Intracellular acting toxins share common modes of action which involves: binding to specific receptors on host cell membranes, internalisation, and interaction with an intracellular target. The intracellular activities vary, but the toxins can be grouped according to their enzymatic and biochemical activities.

### 1.4.2.1 ADP-ribosylating toxins

ADP-ribosylation of eukaryotic proteins is a well established mechanism by which various bacterial toxins affect and kill the host cell. This family of toxins transfers the ADP-ribose moiety of nicotinamide adenine dinucleotide ($NAD^+$) to a target eukaryotic protein, a GTP- or ATP-binding protein. The addition of the bulky ADP-ribose group alters the function of the target protein (Domenighini et al., 1995). ADP-ribosylating toxins can be divided into four groups according to their target proteins. The first group of toxins catalyses the ADP-ribosylation of the eukaryotic elongation factor 2 (EF2), a ribosome interacting protein. The diphtheria toxin of Corynebacterium diphtheriae and the exotoxin A of Pseudomonas aeruginosa transfer ADP-ribose to a post-translationally modified histidine residue, diphthamide, an amino acid unique to EF2. The ADP-ribosylation of EF2 results in inhibition of protein synthesis and eventually leads to cells death (Boquet and Gill, 1991).

The second group of toxins catalyses the ADP-ribosylation of GTP-binding proteins involved in regulation of transmembrane signalling. The cholera toxin of Vibrio cholerae catalyses the transfer of ADP-ribose to the $\alpha$ subunit of the G protein,

$G_s$, the membrane bound positive regulator of adenylate-cyclase. The ADP-ribosylation of $G_{s\alpha}$ is enhanced by an ADP-ribosylation factor (ARF) which can bind GTP and the cholera toxin. ADP-ribosylation of $G_{s\alpha}$ renders it incapable of dissociating from the active cyclase complex, resulting in permanent activation. This leads to an elevated level of cyclic AMP (cAMP) which interferes with ion transport, causing an efflux of ions and then fluid from the cells (Spangler, 1992). The heat-labile enterotoxin of *E. coli* is both structurally and functionally similar to the cholera toxin (Spangler, 1992). The pertussis toxin of the *Bordetella spp.* also belongs to this group of toxins and catalyses the ADP-ribosylation of the G protein, $G_i$ (see Section 1.6.7).

The third group of such toxins are involved in the ADP-ribosylation of the small GTP-binding protein Rho. This molecule belongs to the Ras superfamily (Hall, 1994) (see Chapter 5). The *Clostridium botulinum* exoenzyme C3 catalyses the ADP-ribosylation of the Rho protein, rendering the GTP binding protein inactive. The inactivation of Rho by C3 ADP-ribosylation does not effect the endogenous GTPase cycle of Rho, but inhibits the interaction of the modified Rho with a putative effector (Aktories, 1994; Aktories and Koch, 1995). Treatment with C3 induces rounding and binucleation of Swiss 3T3 cells (Rubin *et al.*, 1988), and depolymerisation of actin in Vero cells (Chardin *et al.*, 1989). Due to its poor cell penetration the role of C3 in pathogenesis is unclear. However, this toxin has provided valuable information about the role of Rho in cytoskeleton reorganisation (Ridley and Hall, 1992). Several other exoenzymes that modify Rho by ADP-ribosylation have been identified, forming a family of C3-like transferases (Aktories and Koch, 1995). This family includes *Clostridium limosum* transferase, *Bacillus cereus* transferase and *Staphylococcus aureus* EDIN (epidermal differentiation inhibitor).

The final group of ADP-ribosylating toxins do not target GTP-binding proteins but ATP-binding proteins. *Clostridium botulinum* C2 toxin catalyses the ADP-ribosylation of monomeric G-actin, resulting in the loss of both its ATPase activity and its ability to polymerise (Aktories, 1994; Aktories and Koch, 1995). The acceptor amino acid for the ADP-ribose is located in the specific area of the G-actin involved in the contact site with polymerised F-actin. Therefore, the bulky ADP-ribose group blocks the formation of polymerised F-actin by steric hinderance, resulting in the destruction of the cellular microfilament network. Other toxins that ADP-ribosylate

actin are *Clostridium perfringens* iota toxin, *Clostridium spiroforme* toxin and an ADP-transferase produced by *Clostridium difficile* (Aktories, 1994; Aktories and Koch, 1995).

## 1.4.2.2 Monoglucosyltransferase

*Clostridium difficile* toxin A and B induce the destruction of the cellular microfilament network and cause similar morphological changes to cells as those induced by exoenzyme C3 (Fiorentini *et al.*, 1989). Unlike C3, toxin A and B are monoglucosyltransferases that cleave the substrate UDP-glucose and transfers the glucose moiety to the Rho protein (Just *et al.*, 1995a; 1995b). Toxin A and B also glucosylates other members of the Rho family, Rac and Cdc42 rendering them inactive. Monoglucosylation is a novel mechanism by which intracellular acting bacterial protein toxins affect their target cells (Aktories and Just, 1995; von Eichel-Streiber *et al.*, 1996). Recently, new members to this toxin group have been identified, *Clostridium sordellii* lethal toxin catalyses glycosylation of GTP-binding proteins Rac and Ras (Just *et al.*, 1996), and *Clostridium novyi* α toxin has been shown to a be an N-acetyl-glucosaminyltransferase that modifies Rho, Ras and Cdc42 (Selzer *et al.*, 1996).

## 1.4.2.3 N-glycosidase

The shiga toxin, produced by *Shigella dysenteriae*, is the prototype for the shiga-like toxins produced by enterohaemorrhagic strains of *E. coli*. These toxins inhibit protein synthesis in eukaryotic cells, leading to cell death by inactivating 60S ribosomal subunits. These toxins are N-glycosidases which cleave the N-glycosidic bond at a specific adenine residue of the 28S rRNA in the 60S subunit ribosome. This results in inhibition of protein synthesis by interfering with the binding of aminoacyl-tRNA to the 60S ribosomal subunit via elongation factor 1 (Endo *et al.*, 1988). Interestingly, the shiga toxin and shiga-like toxins have identical modes of action on eukaryotic ribosomes as the plant toxin ricin (Endo and Tsurugi, 1987).

## 1.4.2.4 Adenylate cyclase

Adenylate cyclase is secreted as a toxin by *Bordetella* species (see Section 1.6.6) and *Bacillus anthracis* which produces edema toxin as part of the anthrax toxin complex. Both toxins are stimulated by calmodulin, a calcium-binding protein supplied by the target cell but absent from the bacteria. The toxins generate uncontrolled levels of intracellular cAMP which inhibits the function of immune effector cells. Many bacteria including *E. coli* produce adenylate cyclase to control the levels of bacterial cAMP. But the adenylate cyclases from *Bordetella* species and *Bacillus anthracis* form a unique class of enzymes which are secreted and activated by a protein produced in the host cell and thereby toxic to eukaryotic cells (Hewlett and Maloney, 1995; Peterkosky *et al.*, 1993).

## 1.4.2.5 Neurotoxins

Tetanus toxin of *Clostridium tetani* and the seven serologically distinct botulinum neurotoxins of *Clostridium botulinum* (designated BoNT/A to BoNT/G) are potent neurotoxins. The tetanus and botulinum neurotoxins are metallo-proteases, requiring a $Zn^{2+}$ ion for catalytic activity. The targets for these toxins are neuronal membrane proteins which are involved in the fusion of synaptic vesicles to the active zone of the presynaptic membranes. The neuronal membrane protein synaptobrevin is proteolytically cleaved by the tetanus toxin, BoNT/B, BoNT/D, BoNT/F and BoNT/G, the protein syntaxin is the target for BoNT/C1, and the protein SNAP25 (synaptosomal associated protein of 25 kDa) is cleaved by BoNT/A and BoNT/E. The toxins that attack synaptobrevin and syntaxin cleave the proteins at different peptide bonds. Proteolytic cleavage inhibits synaptic function, therefore, blocking endocytosis of the synaptic vesicles and release of neurotransmitters. The tetanus toxin blocks the release of inhibitory neurotransmitters in the spinal cord leading to spastic paralysis. The botulinal toxins inhibit the exocytic release of acetylcholine in the peripheral motorneurons leading to flaccid paralysis (Niemann *et al.*, 1994; Montecucco and Schiavo, 1995).

### 1.4.2.6 A new family of toxins

A new family of toxins has been proposed for *Pasteurella multocida* toxin (PMT), the cytotoxic necrotizing toxins (CNF type 1 and 2) of *E. coli* and the dermonecrotic toxin (DNT) of *Bordetella* species (see Section 1.6.4). This family was proposed based on shared sequence homology and shared common characteristics. These toxins are all large polypeptides which affect regulation of cell growth or division. All the toxins are nonsecreted proteins without typical signal sequence (Lax *et al.*, 1990; Falbo *et al.*, 1993; Oswald *et al.*, 1994; Pullinger *et al.*, 1996). Both PMT and the CNFs appear to require cell entry and processing via acidic compartment for activation (Rozengurt *et al.*, 1990; Oswald *et al.*, 1994).

There is limited homology among this family of toxins at the amino acid level. CNF 1 and CNF 2 are 86% identical at the DNA level (Oswald *et al.*, 1994). At the amino acid level there is 55% identity between the N-terminal fragment of the CNFs and PMT, over a 204 amino acid region (Falbo *et al.*, 1993; Oswald *et al.*, 1994). There is a region of significant homology between the CNFs and DNT near their C-termini (Walker and Weiss, 1994; Pullinger *et al.*, 1996). In DNT this region contains a putative nucleotide binding motif, which is essential for DNT activity (Pullinger *et al.*, 1996). But this motif would not be functional in CNF because only the glycine residue of the essential glycine and lysine residues is present in the CNF sequence (Walker and Weiss, 1994). There is no significant homology between DNT and PMT (Walker and Weiss, 1994; Pullinger *et al.*, 1996).

All these toxins stimulate DNA synthesis in quiescent cells. PMT is a potent mitogen causing cell proliferation in the absence of other growth promoting factors (Rozengurt *et al.*, 1990). PMT also induces anchorage independent growth of Rat-1 cells (Higgins *et al.*, 1992). PMT activates inositol phosphate production leading to $Ca^{2+}$ release, suggesting that PMT stimulates the phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphate (Staddon *et al.*, 1991a; Wilson *et al.*, 1997). PMT also increases cellular content of diacylglycerol which activates protein kinase C (Staddon *et al.*, 1990). PMT causes striking rearrangement of the actin cytoskeleton, inducing actin stress fibre and focal adhesion formation, and phosphorylation of $p125^{FAK}$ and paxillin (Lacerda *et al.*, 1996). GTP-binding proteins are involved in PMT toxicity. PMT potentiates the action of bombesin, suggesting $G_q$ is activated (Murphy

and Rozengurt, 1992) and recently, has been shown to activate the inositol phosphate signalling pathway via $G_{q\alpha}$ (Wilson *et al.*, 1997). Also the effect of PMT on the cell cytoskeleton implies the GTP-binding proteins of the Rho family are involved (Lacerda *et al.*, 1996).

DNT and the CNFs also stimulate DNA synthesis in quiescent cells, but unlike PMT, they induce nuclear division without subsequent cell division, resulting in bi- or multi-nucleated cells (Fiorentini *et al.*, 1988; Horiguchi *et al.*, 1993; Oswald *et al.*, 1994). DNT and the CNFs induce the reorganisation of the cell cytoskeleton, resulting in the formation of actin stress fibres and focal adhesions (Fiorentini *et al.*, 1988; Horiguchi *et al.*, 1993; Oswald *et al.*, 1994). The CNF1 actin rearrangement induces phagocytic behaviour in HEp-2 cells, allowing normally non-invasive bacteria to be taken up intracellularly (Falzono *et al.*, 1993). Recently, it has been shown that both CNFs and DNT target and modify the Rho protein (Oswald *et al.*, 1994; Fiorentini *et al.*, 1995; Horiguchi *et al.*, 1995). Unlike other bacterial toxins which are known to modify Rho, DNT and the CNFs modify the Rho protein such that its activity is stimulated (Oswald *et al.*, 1994; Fiorentini *et al.*, 1995; Horiguchi *et al.*, 1995).

The modification of targets catalysed by PMT, DNT and CNFs is unknown. Modification of Rho by CNF2 does not involve NAD-dependant ADP-ribosylation or ATP-dependant phosphorylation of the Rho protein (Oswald *et al.*, 1994). Ward *et al.* (1994) showed that a putative ADP-ribosylation motif is not functional in PMT, which is consistent with the failure to detect ADP-ribosylation activity of PMT using whole cells (Staddon *et al.*, 1991b). A putative nucleotide binding motif in DNT suggests that the toxin may be an ATP-binding protein. The role of this motif is unknown, but it is possible that ATP-hydrolysis could be a source of energy for toxin action. Therefore, PMT, DNT and CNF type 1 and 2 are a group of toxins which modify GTP-binding proteins effecting cell growth or division via novel mechanisms.

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## 1.5 The Genus Bordetella

Until recently, the genus *Bordetella* contained four species, *B. bronchiseptica*, *B. pertussis*, *B. parapertussis* and *B. avium* (Weiss, 1992). These species are all important pathogens in human or veterinary respiratory disease. *B. bronchiseptica* causes atrophic rhinitis in pigs, respiratory infections in domestic and wild animals (Goodnow, 1980) and occasionally in man, especially immunocompromised AIDS patients (Woodard *et al.*, 1995). *B. pertussis* causes human whooping cough (Weiss & Hewlett, 1986), while *B. parapertussis* is associated with a milder form of whooping cough, and has been isolated from healthy and pneumonic lambs (Porter *et al.*, 1994). *B. avium* is pathogenic for birds, causing respiratory disease (rhinotracheitis) in turkeys (Kersters *et al.*, 1984). Three new species have been added to the genus based on DNA hybridisation studies and other genotypic and phenotypic characteristics. The new species *B. holmesii* was proposed for 15 strains isolated from blood cultures of immunocompromised patients (Weyant *et al.*, 1995). Vandamme *et al.* (1995) proposed the new species *B. hinzii* for *B. avium*-like organisms isolated from the respiratory tract of poultry and two humans. Recently, Vandamme *et al.* (1996) proposed the species *B. trematum* for strains isolated from wounds and ear infections in humans.

*Bordetella* species are Gram-negative coccobacilli and obligate aerobes. All possess a respiratory metabolism, are nonfermentative, and produce catalase and lysine dehydrogenase. *Bordetella* species are usually isolated from diseased organisms by taking swabs from nasal or tracheal tracts, but *B. pertussis* is rarely isolated during the final (convalescent) stages of the disease. Pittman (1979) suggested that this failure to culture the bacteria late in disease may be due to their transient presence in the respiratory tract, and that the disease was due to the effects of toxins released early in infection. *B. pertussis* has not been isolated from sources other than human disease and the failure to culture *B. pertussis* during the later stages of pathogenesis may be due to the fact that it is extremely fastidious and slow growing. Alternatively, the inability to culture *B. pertussis* late in disease may be due to a few bacteria being able to survive in a transient intracellular phase. It has been shown that virulent *B. pertussis* can invade and survive inside human epithelial cells (Ewanowich *et al.*, 1989a) and human macrophages (Friedman *et al.*, 1992) *in vitro*. It has been postulated that asymptomatic individuals who carry intracellular *B. pertussis* may

act as a reservoir for this bacterium (Weiss, 1992). *B. parapertussis* and *B. bronchiseptica* can also invade and survive in epithelial cells (Ewanowich *et al.*, 1989b; Savelkoul *et al.*, 1993), but *B. bronchiseptica* was able to invade HeLa cells in both virulent and avirulent phases (Savelkoul *et al.*, 1993; Schipper *et al.*, 1994). Both *B. bronchiseptica* and *B. avium*, unlike *B. pertussis*, can survive and grow in phosphate buffered saline and lakewater suggesting that these species can survive outside the host (Porter *et al.*, 1991; Porter and Wardlaw, 1993).

   *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* are closely related, sharing 72-94% DNA homology (Kloos *et al.*, 1981), and should be considered members of a single species (Musser *et al.*, 1986). However, like many clinically important microorganisms, they have been split into different species. A phylogenetic tree of the genus *Bordetella* was deduced from the nucleotide sequences of the pertussis toxin operon (Aricò *et al.*, 1987) and the presence of insertion sequences (Gross *et al.*, 1989) to show the evolutionary relationship between the *Bordetella* species. The model suggests that *B. avium* diverged from the ancestral *Bordetella* strain before the acquisition of the pertussis toxin gene, and is considered the most distant member of the genus. Phylogenic analysis shows that *B. pertussis* and *B. parapertussis* diverged from *B. bronchiseptica*, corresponding with the loss of motility and specificity for humans. Recently, van der Zee *et al.* (1996) studied DNA polymorphisms associated with repetitive DNA sequences in the *Bordetella* species, and found that ovine and human *B. parapertussis* strains appear to have evolved independently from *B. bronchiseptica* strains and to have adapted to different hosts.

## 1.6 Virulence Factors of the Bordetella Species

   The ability of the *Bordetella* species to cause different diseases in various hosts is probably due to host determinants and the different virulence factors produced by the different species. For example, Tuomanen *et al.* (1983) showed that human pathogens, *B. pertussis* and *B. parapertussis*, adhere better to human ciliated cells than to ciliated cells from rabbits, mice or hamsters. In contrast, *B. bronchiseptica* showed preferential adherence to non-human ciliated cells.

   *Bordetella* species produce a variety of toxins, aggressins and adhesins, all of which are thought to be important in colonisation of their respective hosts and

ensuring survival and propagation of the bacteria. Research on *Bordetella* has focused mainly on the virulence factors of the human pathogen *B. pertussis* (Relman, 1995). However, most of these virulence factors have also been identified in the other *Bordetella* species (Table 1.2) and the close relationship between the species suggests that the virulence factors may play a similar role in the pathogenicity of *B. parapertussis* and *B. bronchiseptica*. The role of the virulence factors in diseases caused by *B. pertussis, B. parapertussis* and *B. bronchiseptica* is discussed below, but the newly described species are not considered, as their virulence factors have not been studied.

### 1.6.1 Filamentous Haemagglutinin

Filamentous haemagglutinin (FHA) is recognised as the major adhesin in *Bordetella* species except *B. avium*, which does not produce FHA (Gentry-Weeks *et al.*, 1988). FHA is associated with the bacterial surface and has been found in culture supernatants. The FHA protein is 220 kDa, though sequence determination of its gene reveals a single open reading frame sufficient to encode a 367 kDa precursor polypeptide (Relman *et al.*, 1989; Domenighini *et al.*, 1990). The mature FHA protein is derived from the N-terminus of this precursor, and other FHA-associated polypeptide species are derived from post-translational proteolytic processing of the 220 kDa protein.

Locht *et al.* (1992) found that the export of FHA is dependent on genes located downstream of the FHA structural gene, *fhaB*, and that this region was also required for the expression of fimbriae. This region has a cluster of open reading frames, designated *fimABCD*, which show sequence homology to genes involved in the export and assembly of fimbriae in Gram-negative bacteria (Locht *et al.*, 1992; Willems *et al.*, 1992). Willems (1994) identified a gene, *fhaC*, downstream of *fimD*, and showed that mutations in *fhaC* abolished the production of FHA, but not fimbriae. The FhaC protein has sequence homology to ShlB and HpmB outer membrane proteins involved in secretion and activation of *Serratia marcescens* and *Proteus mirabilis* haemolysins. Recently, Renauld-Mongénie *et al.* (1996) proposed a model in which the C-terminal domain of the FHA precursor acts as an intramolecular chaperone to prevent premature folding of FHA, keeping the N-terminus of FHA free to interact with FhaC

| Virulence factor | *Bordetella pertussis* | *Bordetella parapertussis* | *Bordetella bronchiseptica* | *Bordetella avium* |
|---|---|---|---|---|
| filamentous haemagglutinin | + | + | + | - |
| fimbriae | + | + | + | + |
| pertactin | + | + | + | - |
| dermonecrotic toxin | + | + | + | + |
| tracheal cytotoxin | + | + | + | + |
| adenylate-cyclase/haemolysin | + | + | + | - |
| pertussis toxin | + | - | - | - |

**Table 1.2** Virulence factors of the *Bordetella* species.

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and be transported out of the cell. Willems *et al.* (1994) suggested that the production of FHA is affected by mutations in the fimbriae genes *fimC* or *fimD*, because *fhaC* is translationally coupled to these genes. The close link between these genes may be a selective advantage, allowing co-regulation of virulence genes that function together in infection.

FHA-negative *B. pertussis* strains were used to show that FHA was necessary for bacterial adherence to ciliated respiratory epithelial cells (Tuomanen and Weiss, 1986). Kimura *et al.* (1990) showed that FHA is important for tracheal colonisation during respiratory infection in mice, but not important for colonisation of the lungs.

FHA has been shown to have three different binding properties. i) From the nucleotide sequence of FHA two Arg-Gly-Asp (RGD) sequences are predicted (Relman *et al.*, 1989), one within the mature 220 kDa protein. RGD sequences are sites of cell recognition for eukaryotic extracellular matrix proteins (Ruoslahti and Pierschbacher, 1986). Relman *et al.* (1990) showed that conservative mutation of the RDG site impaired the ability of the FHA-mediated binding to human macrophages, but not to ciliated epithelial cells, and that adhesion was via the macrophage integrin CR3 ($\alpha_M\beta_2$, CD11b/CD18). ii) FHA has affinity for lactose-containing glycolipids on ciliated cells and macrophages (Tuomanen *et al.*, 1988). iii) FHA has been shown to bind to heparin (Menozzi *et al.*, 1991), a sulphated polysaccharide commonly found in extracellular matrices of eukaryotic cells. Inhibition of sulphation of cellular proteoglycan resulted in reduced *B. pertussis* adherence, suggesting that sulphated glycoconjugates exposed on the cell-surface may serve as receptors (Menozzi *et al.*, 1994). These different binding properties show how FHA can mimic eukaryotic cell adhesion mechanisms for mediating adherence of *Bordetella* species to mammalian cells.

## 1.6.2 Fimbriae

Fimbriae, or pili, are important adherence factors for many bacterial pathogens (de Graaf and Mooi, 1986; Finlay and Falkow, 1989). They are filamentous structures of high molecular weight, and are composed of identical subunits of approximately 20 kDa. All four *Bordetella* species produce a number of serologically related fimbriae subunit proteins of 21-24 kDa (Lee *et al.*, 1986; Mooi *et al.*, 1987). These *Bordetella*

fimbriae subunits share homology with fimbriae subunits from *E. coli, Haemophilius influenzae,* and *Proteus mirabilis* (Mooi *et al.,* 1987). Five major subunit genes have been identified in *B. pertussis; fim2, fim3, fimA, fimX* and *fimY,* though *fimX* and *fimY* are silent (Mooi *et al.,* 1987). Usually, a single gene cluster encodes for the major fimbriae subunits and accessory proteins required for export and assembly of the fimbriae structure (de Graaf and Mooi, 1986). However, the *Bordetella* fimbriae subunits are not located within a single gene cluster. Locht *et al.* (1992) and Willems *et al.* (1992) independently reported a gene cluster containing four fimbriae genes, *fimABCD,* located downstream of *fhaB.* The gene, *fimA,* codes for a structural subunit protein, while *fimB* encodes a fimbriae chaperon-like protein with homology to *Klebsiella pneumoniae* MrkB and *E. coli* PapC. *fimC* codes for a protein which has homology to a class of proteins probably involved in the transport of the fimbriae subunits across the outer membrane (Willems *et al.,* 1992). *fimD* encodes a minor fimbriae subunit with homology to *K. pneumoniae* MrkD, known to be involved in binding to the host receptor (Willems *et al.,* 1993).

A mutant strain of *B. pertussis* lacking both Fim2 and Fim3 was unable to colonise mouse trachea, but was still capable of colonising the mouse nasopharynx and lungs (Mooi *et al.,* 1992). Hazenbos *et al.* (1994; 1995) showed that *B. pertussis* mutant strains lacking both the minor and major fimbrial subunits had reduced adherence to human monocytes, and showed that *B. pertussis* binds to human monocytes with the minor fimbrial subunit FimD. Recently, the major fimbrial subunit of *B. pertussis* was shown to bind to sulphated sugars, and to contain two regions similar to fibronectin peptides which also bind to sulphated sugars (Geuijen *et al.,* 1996).

### 1.6.3 Pertactin

The pertactins are a family of outer membrane proteins which are serologically related in the genus *Bordetella.* They have been named on the basis of their molecular weights: P69 from *B. pertussis,* P70 from *B. parapertussis* and P68 from *B. bronchiseptica* (Montaraz *et al.,* 1985). The sequence of the pertactin gene from *B. pertussis* (Charles *et al.,* 1989), *B. parapertussis* and *B. bronchiseptica* (Li *et al.,* 1992) showed an open reading frame encoding a polypeptide of 93 kDa, considerably

larger than the mature protein. Expression of the *prn* gene in *E. coli* leads to the synthesis of full-length P93 polypeptide, which is rapidly processed to the mature P69 protein located at the cell surface (Charles *et al.*, 1989). Charles *et al.* (1994) showed that the P93 precursor polypeptide is processed at both termini. Thirty-four amino acids are removed from the N-terminus, and a polypeptide of about 30 kDa is cleaved from the C-terminus, which remains associated with the outer membrane but not exposed at the cell surface. Deletion of the 3' region of *prn*, encoding the 30 kDa polypeptide, results in the expression of intracellular P69 protein, suggesting that the 30 kDa C-terminal domain is necessary for the correct location of P69 on the cell surface (Charles *et al.*, 1994) .

Purified pertactin from *B. bronchiseptica* acts as a protective antigen against *B. bronchiseptica* infection in piglets and mice (Montaraz *et al.*, 1985; Novotny *et al.*, 1985). Leininger *et al.* (1991) showed that *B. pertussis* strains which specifically lack pertactin were less efficient at adhering to CHO and HeLa cells than wild-type *B. pertussis*. But Roberts *et al.* (1991) demonstrated no defect in adherence or invasion of HEp2 cells by pertactin negative mutants. The failure of pertactin mutants to block adherence completely implies a role for other adhesins, such as FHA and fimbriae. Pertactin, like FHA, contains an Arg-Gly-Asp (RGD) sequence. Leininger *et al.* (1991; 1992) showed that the adherence of purified pertactin to epithelial cells and the entry of *B. pertussis* into HeLa cells were both inhibited by competitive RGD-containing peptides. Recently, Emsley *et al.* (1996) determined the X-ray crystal structure of pertactin, and showed that it contains structural features that are common to proteins exhibiting binding activities, such as proline-rich regions.

### 1.6.4 Dermonecrotic toxin

Dermonecrotic toxin (DNT) was first identified by Bordet and Gengou in 1909, and is defined on the basis of its biological activity upon intradermal injection into animals. DNT is also known as heat labile toxin because it is inactivated by heat treatment at 56°C (Munzo, 1971). At high doses DNT is lethal to mice (Wardlaw and Parton, 1983). Despite being one of the first reported virulence factors for *B. pertussis*, very little is known about DNT and its role in disease pathogenesis. Some of the properties of DNT have already been discussed in Section 1.4.2.6.

*B. pertussis, B. parapertussis, B. bronchiseptica* and *B. avium* all produce DNTs that are serologically cross reactive (Evans, 1940; Wardlaw and Parton, 1983). DNT is a cytoplasmic protein and is not secreted by the bacteria (Cowell, 1979). It has been purified from *B. pertussis* and *B. bronchiseptica* by several groups (see Chapter 3), and very recently DNT from *B. bronchiseptica* has been cloned and expressed in *E. coli* (Pullinger *et al.*, 1996) (see Chapter 4).

DNT is considered to be an important virulence factor in the development of turbinate atrophy in atrophic rhinitis (see Section 1.8). However, Weiss and Goodwin (1989) argue that DNT may not play an important role in the pathogenesis of whooping cough because *B. pertussis* mutants lacking DNT were as lethal as the wild type strain in infant mice.

DNT induced vasoconstriction of peripheral blood vessels of guinea pig skin *in vivo* (Endoh *et al.*, 1986a) and blood vessels of perfused lung preparations from guinea pigs *in vitro* (Endoh *et al.*, 1986b). DNT had no contractile effect on isolated arteria, deferent canal, or intestinal preparations from guinea pigs, and did not affect the ciliary movement of cultured tracheal rings (Endoh *et al.*, 1986b). Furthermore, DNT had no effect on cultured cardiac or skeletal muscle cells (Nagai *et al.*, 1992a), but had a specific effect on smooth muscle cells in culture (Endoh *et al.*, 1988a; Nagai *et al.*, 1992a), suggesting that the primary target for DNT was smooth muscle cells. Endoh *et al.* (1986a; 1986b) hypothesised that DNT induced vasoconstriction of the peripheral blood vessels, resulting in reduced blood flow to the region, which caused ischemia followed by haemorrhage and the characteristic dermonecrotic lesions. Endoh *et al.* (1988b) suggested that DNT acted on vascular muscle cells by damaging their membrane permeability leading to an increase in $Ca^{2+}$ influx which resulted in contraction. The mode of vasoconstriction induced by DNT appeared to differ from that induced by drugs such as norepinephrine or histamine (Endoh *et al.*, 1986b); injection of pharmacological vasoconstrictors did not produce skin lesions similar to those caused by DNT (Walker and Weiss, 1994). Parton *et al.* (1985; 1986) found that the anti-inflammatory drug, prednisolone, if given before a challenge with DNT, was protective against the lethal effect of DNT and the haemorrhagic effect produced by DNT in mouse skin.

Parton (1986) showed that young mice, 3-5 weeks old, were highly susceptible to DNT, but they became resistant to DNT as they matured. Endoh *et al.* (1990) isolated unsaturated fatty acids from the skin of adult mice, which inhibited the action of DNT. The lipids were identified as linoleic and oleic acids. The content of these fatty acids in the skin correlated inversely with the ability of DNT to cause skin lesions in mice. Nagai *et al.* (1992b) suggested that DNT might affect lipid metabolism in target cells. DNT was shown to cause the release of arachidonic acid and phosphatidylcholine from smooth muscle cells, but DNT does not have intrinsic phospholipase $A_2$ or C activity (Nagai *et al.*, 1990; Nagai *et al.*, 1992b). DNT had no effect on the release of arachidonic acid or phosphatidylcholine from cells at $0°C$ or in the absence of $Ca^{2+}$ (Nagai *et al.*, 1992b). Interestingly, arachidonic acid metabolites are potential mediators of inflammation and vasoconstriction. The anti-inflammatory agent, prednisolone, prevents generation of these arachidonic acid metabolites, thereby preventing the lethal and dermonecrotic actions of DNT (Parton, 1985;1986).

DNT caused splenoatrophy and suppressed *in vivo* antibody response in mice (Horiguchi *et al.*, 1992). DNT did not directly affect the function of T and B lymphocytes from mice *in vitro* (Endoh *et al.*, 1988a), therefore the failure of the lymphocytes to proliferate and differentiate was due to dysfunction of the spleen (Horiguchi *et al.*, 1992). Endoh *et al.* (1986a) suggested that splenoatrophy caused by DNT was due to its vasoconstrictive action on spleen arterioles.

DNT has also been shown to alter the function of osteoblast cells (see Section 1.8) and a possible target for DNT has been identified, the small GTP-binding protein, Rho (see Chapter 5).

### 1.6.5 Tracheal cytotoxin

All *Bordetella* species produce tracheal cytotoxin (TCT), a 921 Da muramyl peptide, which is a natural breakdown product of the *Bordetella* cell wall peptidoglycan (Cookson *et al.*, 1989). TCT is released in the culture supernatant during growth but TCT does not accumulate during stationary phase, suggesting that TCT release is not due to bacterial lysis (Heiss *et al.*, 1995). Although peptidoglycan is common to all Gram-negative bacteria only *Bordetella* species and *Neisseria gonorrhoeae* (Sinha and Rosenthal, 1980) are known to release TCT during growth.

TCT from *N. gonorrhoeae* damages ciliated cells within the fallopian tubes during infection.

TCT caused ciliostasis and ciliated cell destruction of cultured hamster trachea (Goldman *et al.*, 1982), symptoms similar to those seen during *B. pertussis* infection. TCT inhibited DNA synthesis without affecting RNA or protein synthesis in cultured hamster tracheal epithelial cells (Goldman *et al.*, 1982; Cookson *et al.*, 1989). TCT has also been shown to stimulate the production of intracellular cytokine interleukin-1 (IL-1α) in epithelial cells (Heiss *et al.*, 1993), and triggers production of nitric oxide radicals which causes destruction of ciliated cell and prevents division and differentiation of basal cells (Heiss *et al.*, 1994:1995). Inhibitors of nitric oxide synthase prevent the deleterious effects of the toxin.

### 1.6.6 Adenylate-cyclase/haemolysin

Adenylate-cyclase/haemolysin (AC/HA) is a bifunctional protein with both adenylate-cyclase and haemolytic activities (Glaser *et al.*, 1988b). AC/HA is produced by all *Bordetella* species except *B. avium*. AC/HA is associated with the cell surface and is actively secreted by the bacteria as a 200 kDa protein. It penetrates the target host cells and catalyses the production of high levels of intracellular cAMP (Confer and Eaton, 1982) disrupting host cell function. AC/HA is activated by the calcium binding eukaryotic regulatory protein calmodulin.

The link between the adenylate-cyclase and haemolytic activities was established by Weiss *et al.* (1983), who showed that *B. pertussis* Tn-5 mutants lost both activities together. Cloning and sequencing of the *cyaA* structural gene confirmed that both activities were due to a single polypeptide (Glaser *et al.*, 1988a; 1988b). The C-terminus of CyaA has sequence homology to *E. coli* α haemolysin HlyA (Glaser *et al.*, 1988b), and the N-terminus has adenylate-cyclase and calmodulin binding activities (Glaser *et al.*, 1989). Three other genes *cyaB*, *cyaD* and *cyaE* are required for the secretion of AC/HA (Glaser *et al.*, 1988b). Genes *cyaB* and *cyaD* code for proteins which are similar to *E. coli* HlyB and HlyD, which are necessary for the transport and secretion of HlyA across the cell envelope. A fourth gene *cyaC* (Barry *et al.*, 1991) is required for post-translational modification of the toxin. Hewlett *et al.* (1993) showed that *B. pertussis*, with a mutation in *cyaC*, produced AC/HA which

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was enzymatically active but was defective in insertion and transmembrane delivery of the catalytic domain.

Both the adenylate-cyclase and haemolysin activities of AC/HA are required for virulence (Ehrmann *et al.*, 1992; Khelef *et al.*, 1992) and for toxicity, i.e. entry into cells and elevation of cAMP levels (Bellalou *et al.*, 1990). The toxin is thought to penetrate the plasma membrane of target cells, bypassing receptor-mediated endocytosis pathways (Hanski and Farfel, 1985). The exact mechanism of pore formation is unknown, but it is dependant on free calcium ions (Hanski and Farfel, 1985) that induce conformational changes in AC/HA leading to toxic activity (Hewlett *et al.*, 1991). Benz *et al.* (1994) showed that AC/HA forms small ion-permeable channels in lipid bilayer membranes, and experiments with mutant forms of AC/HA suggest that the haemolytic part of the toxin is required for channel formation. It is believed that the haemolytic activity of AC/HA is a mechanism acquired for the delivery of the catalytic domain into the target cell, and it is unclear whether there is an independent role for the haemolysin as a virulence factor (Bellalou *et al.*, 1990; Hewlett and Maloney, 1995).

The catalytic domain is delivered to the cytoplasm, where it is activated by calmodulin and produces uncontrolled levels of cAMP. This intracellular increase in cAMP has a deleterious effect on target cells, particularly cells of the immune system. AC/HA prevents superoxide generation by neutrophils and macrophages in response to soluble stimuli (Confer and Eaton, 1982), and also prevents phagocytic responses in monocytes (Pearson *et al.*, 1987). Masure (1993) showed that AC/HA induced elevation of intracellular cAMP promoted the survival of *B. pertussis* within human macrophages, and suggested that this may be due to the prevention of phagosomal fusion to lysosomes. Khelef *et al.* (1993) demonstrated that AC/HA induced apoptosis in macrophages, and therefore was important for infection, bacterial survival, and escape for the immune response. However, *B pertussis* is unable to survive outside the host, and the intracellular location of *B. pertussis* is thought to be advantageous for pathogenesis, implying that this may be only a limited destruction of host cells.

## 1.6.7 Pertussis toxin

Pertussis toxin is unique to *B. pertussis*. The toxin is a globular, complex protein, composed of five different subunits. The toxin contains one copy of the peptides S1, S2, S3, and S5, and two copies of S4 peptide (Tamura *et al.*, 1982). The toxin has a typical A-B toxin structure, where A subunit (S1 peptide) is enzymatically active, while the B subunit (S2, S3, S4 and S5 peptides) delivers the A subunit to the target cell.

The B subunit binds to glycoproteins and glycolipids on the cell surface (Brennan *et al.*, 1988; van't Wout *et al.*, 1992). The amino acid sequence of the B subunits S2 and S3 peptides is similar to that of C-type lectins (Saukkonen *et al.*, 1992), and mutations in S2 abolish all carbohydrate recognition (Heerze *et al.*, 1992). The crystal structure of the pertussis toxin supports the hypothesis that the amino termini of S2 and S3 are homologous to eukaryotic lectins (Stein *et al.*, 1994), and are possible receptor binding site.

The translocation of the A subunit into the cell is poorly understood, though it is known that reduction of the disulphide bond in the A subunit is essential for catalytic activity (Moss *et al.*, 1983). Subunit A of the pertussis toxin is an ADP-ribosylating toxin like cholera and diphtheria toxins. When active, subunit A catalyses the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD) to the $\alpha$ subunit of the GTP-binding protein, $G_i$, inhibiting its activity. $G_i$ is the inhibitor of the G protein $G_s$, the membrane bound positive regulator of adenylate cyclase. Thereby, pertussis toxin can alter cellular regulation of adenylate cyclase activity and lead to uncontrolled production of cAMP (Katada and Ui, 1982). Pertussis toxin has a remarkably diverse range of biological activities and there is much research in this area. The precise actions of pertussis toxin during infection remains to be determined. However, studies of parapertussis infections show that the symptoms, paroxysmal cough, whooping and vomiting are almost identical to those found in pertussis infections, indicating that pertussis toxin may play only a minor role in causing the typical symptoms of whooping cough (Wirsing von König *et al.*, 1994).

Although pertussis toxin is only produced by *B. pertussis* the gene coding for the toxin is present in *B. parapertussis* and *B. bronchiseptica*, but the gene is not transcribed (Marchitto *et al.*, 1987) due to mutations in the promoter region (Aricò and

Rappuoli, 1987). When the gene for pertussis toxin from *B. pertussis* was introduced into *B. parapertussis* and *B. bronchiseptica*, functional pertussis toxin was produced (Lee *et al.*, 1989), suggesting that there is no post-translational barrier to the expression of this toxin when an intact copy of the gene is present. It is pertinent to consider whether or not pertussis toxin was expressed in the recent common ancestor of the three species. It is possible that mutations causing toxin expression were advantageous and became fixed in *B. pertussis* strains. Alternatively, the change may have occurred in the reverse direction whereby *B. parapertussis* and *B. bronchiseptica* lost pertussis toxin expression due to mutation, leading to the selection of less deleterious strains.

### 1.6.8 Other proposed virulence factors

Most of the virulence factors described above are common to all *Bordetella* species, (see Table 1.2), but some, like the pertussis toxin, are species-specific. It is possible that the common virulence factors play an important role in pathogenesis, whereas the species-specific virulence factors reflect adaptations to a specific host or disease. *B. avium* produces osteotoxin, a bacterial β-cystathionase, which is lethal to osteogenic cells and produces unstable and toxic sulphane sulphur derivatives in the presence of L-cysteine (Gentry-Weeks *et al.*, 1993; 1995). *B pertussis* produces BrkA (Fernadez and Weiss, 1994) and tracheal colonisation factor (tcf) (Finn and Stevens, 1995), both of which show homology to the pertactin precursor, and contain the amino acid RDG motif, also found in FHA and pertactin. Mutants lacking tcf were impaired in their ability to colonise the trachea of mice and a BrkA-negative strain showed reduced adherence to lung fibroblast cells. These results, along with the presence of RDG motifs, suggest that tcf and BrkA play a role in adhesion.

All of the virulence factors described, except TCT, are positively regulated by the *bvg* locus (see Section 1.7). Several *vir*-repressed genes (*vrg*) have been identified which are not expressed when the other virulence factors are expressed (Knapp and Mekalanos, 1988; Beattie *et al.*, 1990). It is possible that these Vrg proteins may play a role in virulence by being expressed either early or late in infection, where they facilitate the survival of the bacteria (see Section 1.7).

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*B. pertussis* and *B. bronchiseptica* acquire iron by means of a siderophore as well as transferrin and lactoferrin binding proteins (Menozzi *et al.*, 1991). Iron acquisition by pathogens is critical within an animal host where iron is a limiting nutrient factor and these factors may be important factors for bacterial survival.

## 1.7 Regulation of virulence factors

Bacterial virulence is dependent on the expression of a variety of virulence factors, which may be required at different stages of infection. Many bacterial pathogens can co-ordinately regulate their virulence factors in response to environmental signals (Stock *et al.*, 1989; Gross, 1993). *Bordetella* species are able to change the expression of sets of virulence factors alternating between virulent and avirulent forms.

The expression of unlinked virulence associated genes is regulated by a central regulatory locus *bvg* (*Bordetella* virulence genes) (Weiss and Falkow, 1984, Coote, 1991). This locus encodes two proteins BvgA and BvgS which have a molecular weight of 23 and 135 kDa respectively (Aricó *et al.*, 1989). These proteins show homology to the 'two component' family of prokaryotic signal transduction proteins comprising of a sensor and regulator (Aricó *et al.*, 1989). Examples include the EnvZ/OmpR system involved in regulation of outer membrane proteins of *E. coli*, the control of chemotaxis in *E. coli* and *Salmonella typhimurium*, and regulation of nitrogen fixation in *Rhizobium meliloti* (Stock *et al.*, 1989; Gross, 1993).

Aricó *et al.* (1989) proposed that BvgS was the signal transmembrane protein which senses external signals, and the signal is transmitted via phosphorylation to BvgA, the cytoplasmic DNA-binding regulator protein (see Figure 1.1). Experimental data confirm this 'two component' regulator theory predicted from the initial sequence data. Stibitz and Yang (1991) showed that BvgS was a transmembrane protein, while BvgA was present in the cytoplasm. Miller *et al.* (1992) showed that mutations in the linker region between the transmembrane and cytoplasmic domain of BvgS abolished sensitivity to environmental modulating signals, providing evidence that BvgS does mediate the response to environmental stimuli. BvgA contains a helix-turn-helix motif, common in other proteins which bind to DNA (Scarlato *et al.*, 1993), and has been

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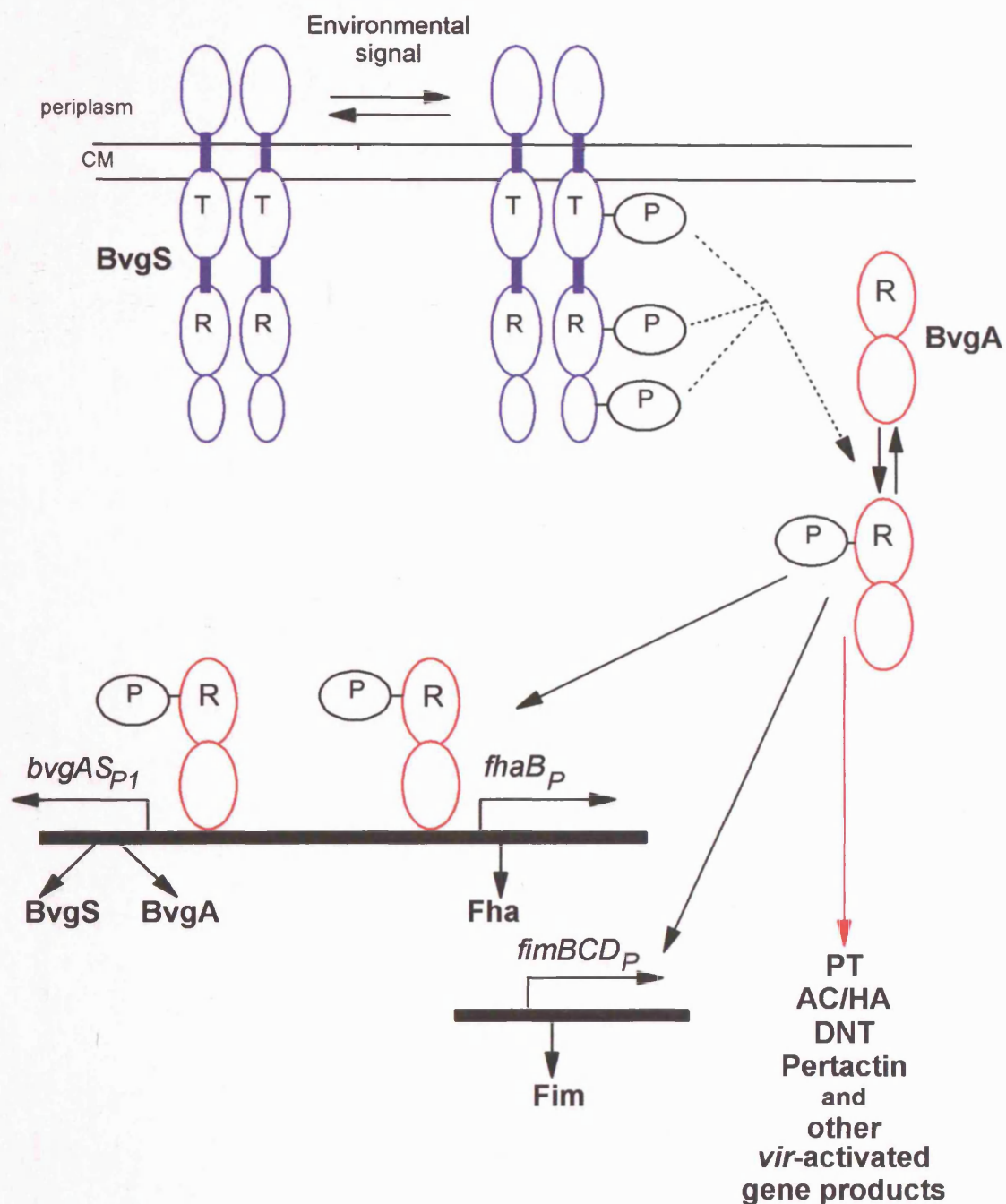
**Figure 1.1** A schematic model of the *Bordetella* species Bvg regulon. CM, bacterial cytoplasmic membrane; T, transmitter protein domain; R, receiver domain; P, phosphorylated residue; subscript p, transcriptional promoter. Thick black horizontial lines indicate regions of chromosome, black solid arrows indicate direct activation of gene expression by BvgA, dotted arrows indicate activation of BvgA, and red solid arrow indicates indirect activation of gene expression by BvgA. See text for further details. Modified from Relman (1995).

shown to bind specifically to the promoter DNA sequences of the *bvg* and *fha* loci (Roy and Falkow, 1991).

The nucleotide sequences of the *bvg* loci of *B. pertussis, B. parapertussis* and *B. bronchiseptica* are highly conserved, especially in the regions coding for the domains involved in kinase and DNA-binding activities (McGillivrary *et al.*, 1989, Aricò *et al.*, 1991). *B. avium* does not contain a genetic locus which is detectable by DNA hybridisation to *B. pertussis bvgA*, but does show DNA homology to *bvgS* (McGillivrary *et al.*, 1989; Gentry-Weeks *et al.*, 1991). However, *B. avium* can regulate the expression of virulence factors, so it is probable that *B. avium* contains a gene which is functionally homologous to *BvgA*.

Many of the *Bordetella* virulence factors require the *bvg* locus for expression. Expression of FHA, DNT, pertussis toxin (Weiss *et al.*, 1983), AC/HA (Laoide and Ullmann, 1990), fimbriae (Willems *et al.*, 1990), and pertactin (Charles *et al.*, 1989) are all regulated by the *bvg* locus. However, TCT expression appears to be *bvg*-independent (Cookson *et al.*, 1989).

A set of *vir*-repressed genes (*vrg*) has been identified which has optimal expression in the presence of modulating signals, when the expression of *vir*-activated genes (*vag*) is reduced (Knapp and Mekalanos, 1988; Beattie *et al.*, 1990). Five *vrg* genes have been identified, and at least one of these, *vrg6*, is important for colonisation of trachea in respiratory infection of mice (Beattie *et al.*, 1990; 1992). Akerley *et al.* (1992) showed that the flagella of *B. bronchiseptica* are encoded by a *vrg* gene. Mutant strains that expressed flagella when the *vag* genes are expressed, i.e. when motility is not normally produced, resulted in a defective tracheal colonisation (Akerley *et al.*, 1995). Therefore, the *Bordetella bvg* locus can act as a positive and negative regulator, and can promote virulence by activating genes required for colonisation, and repress genes that inhibit development of infection.

### 1.7.1 Antigenic Modulation

Antigenic modulation, a form of phenotypic change, controls the reversible expression of virulence factors in response to environmental stimuli. This results in the virulent X mode changing to the avirulent C mode (Lacey, 1960). *In vitro* the most common modulating factors which turn off the expression of *Bordetella*

virulence factors are low temperature (25°C), magnesium sulphate (50 mM) and nicotinic acid (10 mM).

BvgS is a transmembrane protein with a periplasmic region, which senses the environmental stimuli, and several cytoplasmic domains, the linker, transmitter, receiver and an additional C-terminal domain. The mechanism by which the periplasmic sensor domain is able to regulate the activity of the C-terminal transmitter domain is unknown. The BvgS linker region is essential for signal transduction (Miller et al., 1992; Goyard et al., 1994). In the absence of modulators, BvgS is autophosphorylated at a conserved histidine residue in the transmitter domain, and BvgS then transfers the phosphate to a conserved aspartic acid on the receiver domain (Uhl and Miller, 1994). Autophosphorylation of BvgS is required for the phosphotransfer to BvgA (Uhl and Miller, 1994). Recently, Uhl and Miller (1996) showed that the C-terminal domain is an essential part of the phosphotransfer from BvgS to BvgA. It is not known why the three domains of BvgS are necessary for phosphotransfer to BvgA, because in the majority of 'two component' systems a transmitter domain is sufficient for autophosphorylation and phosphorylation of the receiver domain. It is possible that the extra domains in *Bordetella* species may act as control points that sense the intensity or duration of the environmental stimuli.

The expression of the *bvg* locus is regulated by four promoters, $P_{1-4}$ (Scarlato et al., 1990). The $P_1$, $P_2$ and $P_3$ promoters transcribe the BvgA and BvgS proteins, while the $P_4$ promoter transcribes an antisense RNA (Scarlato et al., 1990). The *bvg* locus is activated autogenenously (Roy et al., 1990). The $P_2$ promoter is constitutively active, and low levels of BvgA and BvgS are transcribed from $P_2$ even in the presence of modulators. After a positive signal, BvgS activates BvgA by phosphorylation, which then activates transcription from the $P_1$, $P_3$ and $P_4$ promoters. This results in a 50-fold increase of BvgA proteins levels, leading to the activation of other *bvg* regulated genes (Scarlato et al., 1990; Roy et al., 1990). The $P_1$ and $P_2$ promoters are linked and so binding of BvgA to activated $P_1$ represses transcription of $P_2$ by making the $P_2$ promoter inaccessible to RNA polymerase (Roy and Falkow, 1991). In the presence of modulators the transcription of virulence genes is turned off within six minutes while transcription of the *bvg* locus is maintained for several hours (Scarlato and Rappuoli, 1991). Without phosphorylation of BvgA by BvgS, BvgA becomes

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inactive and unable to bind to the $P_1$ promoter, leading to derepression of the $P_2$ promoter, and constitutive low levels of BvgA are expressed (Scarlato *et al.*, 1990; Roy and Falkow, 1991; Scarlato and Rappuoli, 1991). BvgS levels remain constant and so changes in environmental conditions can always be detected (Stibitz and Yang, 1991).

BvgA directly activates the expression of the *fha* and *bvg* operons (Roy *et al.*, 1989; 1990). Deletions within a specific seven base repeat upstream of both the *fhaB* promoter and the positively-regulated $P_1$ promoter abolished *fhaB* and *bvgAS* transcription (Roy and Falkow, 1991). DNAase-protection analysis showed that BvgA-DNA interactions occurred at these regulatory regions upstream of the *fhaB* and *bvgA* promoters (Roy and Falkow, 1991). The binding recognition sequence was conserved in *B. parapertussis* (Scarlato *et al.*, 1991b), and a similar recognition sequence was identified upstream of *fimB* (Willems *et al.*, 1992).

Recently, it has been shown that BvgA is sufficient for activation of the *B. pertussis* pertussis toxin operon in *E. coli* (Uhl and Miller, 1995). Boucher and Stibitz (1995) showed that BvgA bound specifically to a region upstream of the *ptx* promoter. This binding was strictly dependent on the phosphorylation of BvgA, and binding occurred synergistically with binding of RNA polymerase. The phosphorylated form of BvgA has also been shown to bind specifically upstream of the *cyaA* gene (Karimova *et al.*, 1996). BvgA is a highly versatile transcription factor, regulating its own expression and that of a variety of other virulence genes.

In the presence of environmental stimuli, such as a temperature shift from 25°C to 37°C, the *bvg* regulated promoters can be divided into two groups. Within a few minutes of induction, the *bvg* locus activates expression from its own autoregulated promoter $P_1$, and promoters for *fhaB* and *fimABCD*. The second step occurs several hours later and involves activation of *ptx* and *cyaA* promoters at higher levels of BvgA (Scarlato *et al.*, 1991a). Zu *et al.* (1996) analysed the DNA-binding properties of BvgA on different early- and late-activated promoters. The early and late promoters were recognised with high and low affinity by phosphorylated BvgA, respectively. Early promoters can bind non-phosphorylated BvgA, but binding is enhanced by phosphorylation of BvgA. The late-activated promoters are only recognised by

phosphorylated BvgA. Consequently, the adhesins that are needed early during infection are expressed before the toxins that are required later in infection.

## 1.7.2 Phase variation

Phase variation, or genotypic change, occurs by genetic mutation of the *bvg* locus, in which virulent (phase I) bacteria simultaneously lose all virulence factors and become avirulent (phase III or IV) (Peppler, 1982; Weiss and Falkow, 1984). Phase variation also occurs *in vivo*; phase III *B. bronchiseptica* were isolated several days after infection of pigs with phase I bacteria (Collings and Rutter, 1985) and avirulent phase variants have been isolated during the late stages of pertussis infection (Kasuga *et al.*, 1954). Phase variation can occur in the reverse direction, from avirulent phase to virulent phase, *in vitro* (Weiss and Falkow, 1984) and *in vivo* (Collings and Rutter, 1985). Avirulent phase variants arise in a population at a frequency of $10^{-3}$-$10^{-6}$ depending on the strain (Weiss and Falkow, 1984; Lax, 1985). The expected frequency of random mutation in a bacterial gene is $10^{-6}$ per cell per generation, suggesting that phase variation may be caused by a random mutation followed by selection by *in vitro* conditions (Lax, 1985).

Analysis of four phase variants from *B. pertussis* showed that this avirulent phase resulted from a frame shift mutation caused by the insertion of an extra cytosine residue in a run of six cytosines in the *bvg* locus (Stibitz *et al.*, 1989). Phase variation in *B. pertussis* may also result from insertions (McGillivrary *et al.*, 1989) and deletions (Ward *et al.*, 1992) in the *bvg* locus.

## 1.7.3 Role of regulation in pathogenesis

The role of the complex *bvg* regulatory system in *Bordetella* species *in vivo* is unknown. Antigenic modulation and phase variation have been shown to occur during infection (Kasuga *et al.*, 1954; Collings and Rutter, 1985). The avirulent phase III antigens are less immunogenic than antigen expressed in the virulent phase (Lacey, 1960; Beattie *et al.*, 1992). This would be advantageous to the bacteria, especially during the early stages of infection in avoiding activation of host immune defences and elimination from the host. It would be beneficial also in the late stages

of infection, when the host immune responses are maximal and there would be selective pressure favouring phase variants that no longer express *vir*-activated genes.

The two identified *vir*-repressed genes code for Vrg6 and flagella, indicating that the *vir*-repressed genes are involved in motility and adherence. Expression of these genes would be advantageous early in infection to allow movement to, and attachment at, a new site. This would be very effective in a non-immunogenic form which could subsequently express adhesins and toxins at the new site of infection. Expression of flagella in *B. bronchiseptica* interferes with virulence, and the flagella genes must be repressed to establish a successful infection (Akerley *et al.*, 1995), implying that antigenic modulation is required for pathogenesis.

*Bordetella* species can invade eukaryotic epithelial cells and macrophages *in vitro* (Ewanowich *et al.*, 1989a; 1989b; Friedman *et al.*, 1992; Savelkoul *et al.*, 1993; Schipper *et al.*, 1994). The bacteria can survive intracellularly, protected from the host immune responses. The role of *bvg* regulation in invasion and intracellular survival is unknown, though certain virulence factors are required for uptake into the cell. *B. bronchiseptica*, unlike *B. pertussis* and *B. parapertussis*, can invade HeLa cells in the avirulent phase, which may suggest a role for flagella in this process (Savelkoul *et al.*, 1993). A *B. bronchiseptica bvg* mutant was able to invade and survive within host cells for at least a week (Schipper *et al.*, 1994), suggesting that *bvg*-regulated virulence factors do not play a role in this process. However, AC/HA seems to be important for intracellular survival of *B. pertussis* (Masure, 1992), and so it would appear that intracellular uptake and survival differs between *B. pertussis* and *B. bronchiseptica*.

The relevance of phase variation in pathogenesis may be dependent on the type of mutations and how easily the mutations are reversed. Therefore, antigenic modification may be more significant *in vivo* than phase variation.


## 1.8 Atrophic rhinitis

Atrophic rhinitis is a respiratory disease of pigs found throughout the world in areas of intensive pig rearing. The disease is characterised by atrophy of the turbinate bones, and poor growth rates. Other clinical signs include sneezing, nasal discharge

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and pneumonia. In severe outbreaks all these symptoms are present, while in some herds a mild degree of turbinate atrophy may be the only manifestation of the disease. Atrophic rhinitis is an economically important disease due to decreased growth rates and increased susceptibility to other diseases (Pedersen and Barfod, 1981).

Atrophic rhinitis was first described in 1830 by Franque in Germany. Since then there has been much controversy about the etiology of the disease. Various etiological agents and factors have been associated with atrophic rhinitis, including genetic defects, nutritional deficiencies, and infectious agents. Now, there is good evidence that *B. bronchiseptica* and *P. multocida* are the principle causes of atrophic rhinitis (Rutter, 1985).

*B. bronchiseptica* was first associated with atrophic rhinitis by Switzer (1956). But the role of *B. bronchiseptica* in the disease was difficult to establish because it could be isolated from nasal swabs of herds with atrophic rhinitis and also from unaffected herds (Rutter, 1981). Also, experimental infection of pigs with *B. bronchiseptica* alone, caused mild, non-progressive atrophy of the turbinate bones, but clinical atrophic rhinitis did not develop and the turbinate bones could regenerate (Pedersen and Barfod, 1981; Rutter and Rojas, 1982; Rutter *et al.*, 1982). De Jong *et al.* (1980) and Rutter and Rojas (1982) demonstrated that infection of pigs with toxigenic *P. multocida* caused severe turbinate atrophy, which was non-regenerating, providing that a sufficiently large number of *P. multocida* were present in the nasal cavity. To achieve the large numbers of *P. multocida* required, the nasal cavity was pretreated with acetic acid (Elling and Pedersen, 1985), or preinfected with *B. bronchiseptica* (Rutter and Rojas, 1982; Rutter, 1983). It was concluded that atrophic rhinitis was caused by a mixed infection of *B. bronchiseptica* and *P. multocida* (Pedersen and Barfod, 1981; Rutter and Rojas, 1982; Rutter, 1983). Infection of pigs with *B. bronchiseptica* is widespread, and so it may be a commensal species that causes disease as an opportunistic pathogen in compromised animals, or it may predispose animals to secondary infections (Woolfrey and Moody, 1991). Interestingly, ovine *B. parapertussis* has been shown to predispose mice to subsequent infection with *Pasteurella haemolytica*, which is regarded as the main agent of acute ovine pneumonia, despite the difficulty in reproducing the disease with the bacteria alone (Porter *et al.*, 1995).

The *P. multocida* toxin (PMT) and DNT from *B. bronchiseptica* have both been shown to play an important role in turbinate atrophy. Intraperitoneal injection of partially pure PMT or recombinant PMT, into pigs produced severe turbinate atrophy, demonstrating the importance of PMT in the pathogenesis of atrophic rhinitis (Chanter *et al.*, 1986; Lax and Chanter, 1990). The role of DNT in the etiology of atrophic rhinitis was first proposed by Hanada *et al.* (1979), who showed that turbinate atrophy in pigs could be caused by an intranasal inoculation of a cell free sonicated extract from *B. bronchiseptica*. Collings and Rutter (1985) demonstrated a positive correlation between mouse lethality of *B. bronchiseptica* cell extracts, i.e. DNT activity, and the ability of those strains to produce turbinate atrophy. In addition, Roop *et al.* (1987) showed that the ability of *B. bronchiseptica* strains to produce turbinate lesions in neonatal piglets was linked with production of DNT. *B. bronchiseptica* strain PV6 is a naturally occurring mutant which produces all virulence factors except DNT (Magyar *et al.*, 1988). In experimental infection of gnotobiotic pigs, PV6 colonised the nasal cavity in large numbers, like cytotoxic phase I strains, but was unable to cause turbinate atrophy (Magyar *et al.*, 1988), providing further evidence that DNT has an important role in turbinate atrophy. Éliás *et al.* (1992) suggested that *B. bronchiseptica* DNT-induced mucosal injury was a precondition of the growth of *P. multocida* in the nasal cavity.

In atrophic rhinitis, the only bone tissue affected by PMT and DNT is the nasal cavity. The turbinate bones are considered to be the fastest growing bone tissue in piglets and therefore, the toxins may only affect fast growing bones (Elling and Pedersen, 1985). PMT causes bone resorption with an increase in the number of osteoclasts in affected bones (Kimman *et al.*, 1987; Dominick and Rimler, 1988; Felix *et al.*, 1992). Recently, PMT has been shown to be a mitogen for primary chicken osteoblasts and inhibits osteoblast differentiation, therefore preventing normal osteoblast development (Mullan and Lax, 1996). In contrast to PMT, the ultrastructural changes of turbinates induced by *B. bronchiseptica* suggest impaired osteoblastic function (Silveira *et al.*, 1982). DNT has been shown to cause morphological changes and multinucleation of a clonal osteoblast MC3T3-E1 cells (Horiguchi *et al.*, 1991). DNT reduced the accumulation of type 1 collagen and alkaline phosphatase, suggesting that DNT acted at the osteoprogenitor stage impairing differentiation into

osteoblasts (Horiguchi *et al.*, 1991). DNT also induced DNA and protein synthesis in these cells without cell proliferation (Horiguchi *et al.*, 1993; 1994). DNT and PMT interfere with the differentiation of osteoclasts and osteoblasts resulting in altered bone remodelling.

## *1.9 Aims*

It has been suggested that PMT and DNT play an important role in the pathogenesis of atrophic rhinitis (Éliás *et al.*, 1992). Cloning and sequencing of the gene encoding PMT (Lax and Chanter, 1990), and purification of PMT (Chanter *et al.*, 1986), allowed the toxin to be characterised and its mechanism of action to be elucidated. In contrast, relatively little is known about DNT due to the fact that it has not been purified.

The aims of this work are:

- Improve the purification of DNT from *B. bronchiseptica*, based on the purification scheme devised by Garrod (1994).

- Devise a robust purification scheme for recombinant DNT.

Using these purified preparations the aims are:

- Characterise recombinant DNT.

- Investigate how DNT interacts with cells to stimulate morphological changes and stimulate DNA synthesis.

- Investigate the uptake of DNT into cells.

These findings will help us to understand the role of DNT in disease.

*Chapter 2*

*Materials and Methods*

## 2.1 Safety

B. bronchiseptica can be a human pathogen and appropriate care was taken to avoid exposure to aerosols. DNT is a potentially lethal toxin; all cell extracts were handled in class 2 microbiological safety cabinets, as required by the Advisory Committee for Dangerous Pathogens. All work with E. coli containing the B. bronchiseptica DNT gene was carried out at containment category 3+, the High Containment Laboratory, as require by the Advisory Committee for Genetic Manipulation.

## 2.2 Chemicals

All chemicals were from BDH unless otherwise stated.

## 2.3 Bacteriological methods

### 2.3.1 Bacterial strains

The porcine B. bronchiseptica strains B58 and PV6 were isolated in Hungary by T. Magyar (Magyar et al., 1988). Strain B58 was isolated from a pig herd with clinical atrophic rhinitis. Strain PV6 was isolated from a herd in which clinical atrophic rhinitis had never been observed. B. pertussis Tohama (DCH8A) and B. pertussis Wellcome 28 (W28G) were phase I strains from the National Institute for Biological Standards and Controls. Table 2.1 lists twenty six B. bronchiseptica strains isolated from dogs, rabbits, pigs, humans or horses provided by N. Guiso (Institut Pasteur, Paris).

E. coli strain XL1-blue/bluescript (pDNT1), known as DNT1, expressed recombinant B. bronchiseptica DNT (rDNT) (Pullinger et al., 1996). The DNT gene of B. bronchiseptica contains a purine nucleotide-binding motif. The possible role of this nucleotide-binding motif of DNT was investigated by site-directed mutagenesis of the conserved lysine residue to alanine. The resulting E. coli strain XL1-blue/bluescript (pK1310A), known as K1310A, expressed mutant rDNT which was inactive (Pullinger et al., 1996).

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| B. bronchiseptica strain | Origin | Reference |
|---|---|---|
| 286 | dog | |
| 11 | dog | Gueirard & Guiso, 1993 |
| 335 | dog | |
| CIP 52125 | dog | |
| 9.73 | rabbit | Gueirard & Guiso, 1993 |
| LC2 | rabbit | Gueirard et al., 1995 |
| LAPR | rabbit | Gueirard & Guiso, 1993 |
| CIP 55110 | rabbit | |
| LC3 | rabbit | |
| 46093 | pig | |
| 12 | pig | Gueirard & Guiso, 1993 |
| 5 | pig | Gueirard & Guiso, 1993 |
| 374 | pig | |
| Bg1 | pig | |
| DEL | human | Gueirard & Guiso, 1993 |
| DANG | human | |
| VAL | human | |
| ALI | human | |
| R1 | human | Gueirard et al., 1995 |
| SEI | human | Gueirard & Guiso, 1993 |
| CVE | horse | |
| CVH | horse | |
| CV1 | horse | |
| CV2 | horse | |

**Table 2.1** *B. bronchiseptica* strains provided by Dr. N. Guiso (Institut Pasteur, Paris) for use in this study.

### 2.3.2 Culture conditions

*Bordetella* strains were stored at -70°C as cell suspensions in Hornibrook broth (Wardlaw *et al.,* 1976) with 15% v/v glycerol. When required, bacteria were spread onto Bordet-Gengou agar (Bordet and Gengou, 1906) plates, incubated at 37°C for 48 hours, then stored at 4°C for a week. The phase of the bacterial strains was visualised on Bordet-Gengou agar by their different colony morphology. Phase I strains produced small domed haemolytic colonies, whereas phase III strains produced large, flat, non-haemolytic colonies.

A single colony, grown on Bordet-Gengou agar, was inoculated into Hornibrook broth and grown overnight at 37°C by shaking in an orbital incubator at 200 rpm. *B. pertussis* strains were inoculated into Stainer and Schölte media (Stainer and Schölte, 1971) and incubated for 48 hours at 37°C, with shaking at 200 rpm.

*E. coli* strains were stored as cell suspensions at -70°C in Luria-Bertani (LB) broth (Sambrook *et al.,* 1988) with 12% (v/v) glycerol. When required, strains were grown in LB broth or 2× YT media (Sambrook *et al.,* 1988), containing ampicillin (20 µg ml$^{-1}$) and tetracyclin (10 µg ml$^{-1}$), at 37°C, shaking at 200 rpm.

The composition of all culture media used is shown in Appendix A.

### 2.3.3 Determination of viable count

A ten-fold serial dilution of bacterial cells grown in liquid medium was made in peptone water. Diluted samples of 100 µl were spread, in duplicate, onto blood agar plates for *Bordetella* strains and LB agar for *E. coli* strains, and incubated at 37°C. After 48 hours the number of colonies was counted.

## 2.4 Partial purification of B. bronchiseptica DNT

This method is based on the partial purification of DNT by Garrod (1994). At all steps the buffers and solutions were cooled to 4°C before use and samples were stored at 4°C when necessary.

### 2.4.1 Preparation of bacterial extracts

A single colony of *B. bronchiseptica* B58 was inoculated into 15 ml of Hornibrook broth, and shaken at 200rpm in an orbital incubator for 18 hours at 37°C. From this culture 10 ml was used to inoculate 1 litre Hornibrook broth, which was incubated for 18 hours at 37°C, shaking at 200rpm. The bacteria were harvested by centrifugation for 20 minutes at 8,000 ×$g$, and resuspended in 200 ml 50 mM Tris/HCl, pH 7.5.

The bacterial suspension was sonicated in 100 ml aliquots at 70W output, using a Branson Sonifer B-52, for 20 x 2 minute pulses, interspersed with 2 minute intervals for cooling on salted ice. The sonicated extract was centrifuged for 45 minutes at 12,000 ×$g$, and the supernatant was filtered through a membrane filter (0.22 μm pore size) and stored at 4°C.

### 2.4.2 DEAE-Sephacel chromatography

A DEAE-Sephacel (Pharmacia) column (12 cm × 1.6 cm) was equilibrated in 50 mM Tris/HCl, pH 7.5 (column buffer). The bacterial extract was loaded on to the column (pump speed 0.8 ml hr$^{-1}$). The loaded column was washed with 2 volumes of column buffer, and eluted with a 400 ml gradient of 0-600 mM NaCl in column buffer. Fractions of 5 ml were collected and assayed for protein by absorbance at 280nm and by 8% SDS-PAGE, and for cytotoxicity on EBL cells.

The fractions from the DEAE-Sephacel column which were cytotoxic for EBL cells and showed a band of approximately 145 kDa by SDS-PAGE were pooled.

### 2.4.3 Hydroxylapatite chromatography

The pooled fractions obtained from DEAE-Sephacel chromatography were diluted with 2 volumes of cold distilled water and applied to a hydroxylapatite (Bio-Rad) column (3 cm × 1.6 cm), previously equilibrated in 10 mM potassium phosphate, pH 7.5 (pump speed 0.6 ml h$^{-1}$). The column was eluted with a 50 ml gradient of 10-250 mM potassium phosphate, pH 7.5. Fractions of 1.5 ml were collected and assayed for protein by absorbance at 280nm and by 8% SDS-PAGE, and for cytotoxicity on EBL cells.

The fractions from the hydroxylapatite column which were cytotoxic for EBL

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cells and showed a band of approximately 145 kDa by SDS-PAGE, were pooled and stored at 4°C. These fractions were referred to as partially purified DNT.

## 2.5 Partial purification of rDNT from DNT1

The method described below has been developed as part of this thesis for the purification of rDNT from DNT1 based on the partial purification of wild-type DNT (Garrod, 1994).

### 2.5.1 Preparation of bacterial extracts

DNT1 from -70°C stock was inoculated into 10 ml of 2 × YT broth with ampicillin at a final concentration of 20 µg ml$^{-1}$ and tetracyclin at 10 µg ml$^{-1}$, and shaken at 200 rpm in an orbital incubator for 16 hours at 37°C. From this culture 5 ml was used to inoculate 300 ml 2 × YT broth, containing ampicillin and tetracyclin, which was incubated for 13 hours at 37°C, shaking at 200rpm. The bacteria were harvested, by centrifugation for 15 minutes at 8,000 ×g, and resuspended in 100 ml 50 mM Tris/HCl, pH 7.5.

The bacterial suspension was sonicated at 70W output, using a Branson Sonifer B-52, for 2 × 2 minute pulses, interspersed with a 2 minute interval for cooling on salted ice. The sonicated extract was centrifuged for 20 minutes at 12,000 ×g, and the supernatant treated with DNAase and RNAase (Sigma), each at 10 µg ml$^{-1}$, for 2 hours at 37°C. The sonicated extract was centrifuged at 12,000 ×g for 20 minutes and the supernatant passed through 0.22 µm filter. The DNT1 sonicate extract was removed from the category 3+ laboratory via the dunk tank, and further purification was carried out in a class 2 safety cabinet at containment category 2.

### 2.5.2 DEAE-Sephacel chromatography

A DEAE-Sephacel (Pharmacia) column (12 cm × 1.6 cm) was equilibrated in 50 mM Tris/HCl, pH 7.5 (column buffer). The bacterial extract was loaded on to the column (pump speed 0.8 ml h$^{-1}$) and the loaded column was washed with 2 volumes of column buffer. The column was eluted with a stepwise gradient of 0-300 mM NaCl in column buffer, 10 ml aliquots of 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300

mM NaCl in 50 mM Tris/HCl pH 7.5 were sequentially loaded on to the column. Fractions of 5 ml were collected and assayed for protein by absorbance at 280nm and by SDS-PAGE and for cytotoxicity on EBL cells.

The fractions from the DEAE-Sephacel column which were cytotoxic for EBL cells and showed a band of approximately 145 kDa by SDS-PAGE were pooled.

### 2.5.3 Hydroxylapatite chromatography

The pooled fractions containing rDNT from the DEAE-Sephacel chromatography were loaded onto and eluted from the hydroxylapatite column as described for the purification of wild type DNT in Section 2.4.3.

The fractions from the hydroxylapatite column which were cytotoxic for EBL cells and showed a band of approximately 145 kDa by SDS-PAGE, were pooled and stored at 4°C. These fractions were referred to as partially purified rDNT.

## 2.6 Other protein purification methods

### 2.6.1 Precipitation of nucleic acids with Kurimover C-7

Kurimover C-7 solution (Kurita Water Industries Ltd. Japan), pH 6, was prepared according to the manufacturer's instructions and added to the sonicated cell extract at a final concentration of 0.25% (w/v). The mixture was stirred for 10 minutes and then centrifuged for 30 minutes at 10,000 ×g. The supernatant was assayed for protein by SDS-PAGE and for cytotoxic activity on EBL cells.

### 2.6.2 Preparation of dialysis tubing

Dialysis tubing was prepared by boiling for 10 minutes in 500 ml of 2% (w/v) sodium bicarbonate and 1 mM EDTA. The dialysis tubing was cooled to room temperature, washed thoroughly with distilled water and stored at 4°C.

### 2.6.3 Medium pressure gel filtration column chromatography

This work was carried out in collaboration with Jim Sinnet-Smith (ICRF, Lincoln Inn Field, London). A pre-packed Superose 12 gel filtration column (Pharmacia) (5 cm × 1.5 cm) was equilibrated with 50 mM phosphate buffer, pH 7.1,

containing 1 M urea and 0.3 M Na$_2$SO$_4$. The column has a fractionation range of 1-300 kDa.

Fractions from the hydroxylapatite column containing DNT were dialysed overnight against 50 mM phosphate buffer, pH 7.1, containing 1 M urea and 0.3 M Na$_2$SO$_4$. The dialysed sample was concentrated 10-fold using a Centricon-50 concentrator (Amicon). The concentrated sample, 200 μl, was loaded onto the Superose 12 column. The column was eluted with 50 mM phosphate buffer, pH 7.1, containing 1 M urea and 0.3 M Na$_2$SO$_4$. Fractions of 1 ml were collected, and assayed for protein by SDS-PAGE.

### 2.6.4 Preparative isoelectric focusing

Isoelectric focusing (IEF) was performed using the Rotofor cell (Bio-Rad). The DNT samples from DEAE-Sephacel chromatography or hydroxylapatite chromatography were dialysed for 6 hours against distilled water to reduce the concentration of salts. Carrier ampholytes (Bio-lytes, Bio-Rad), covering the pH range 3-10, were added to the sample at a final concentration of 2% (w/v). The mixture was made up to 50 ml with distilled water and loaded into the Rotofor. The sample was bought down to running temperature (4°C) by rotating the Rotofor for 15 minutes before applying the electric current. The samples were run at a constant power of 12 watts. After about 4 hours the run was complete, and when the voltage was stable, the fractions were collected, their pH values measured, and aliquots analysed by SDS-PAGE.

### 2.6.5 Preparation of DNT1 cell lysates

DNT1 from a -70°C stock was inoculated into 10 ml of 2 × YT broth with ampicillin and tetracyclin, and shaken at 200 rpm in an orbital incubator for 16 hours at 37°C. This culture was used inoculate 300 ml 2 × YT broth containing ampicillin and tetracyclin. The culture was incubated for 13 hours at 37°C with shaking. The cells were harvested by centrifugation at 8,000 ×g for 20 minutes. The supernatant was decanted, and the cell pellets resuspended and pooled in 150 ml lysis buffer (50 μg ml$^{-1}$ lysozyme, 5 mM EDTA, 0.1% (v/v) toluene, 50 mM Tris/HCl pH 7.0, 100 mM NaCl) and left to stand at room temperature for 4 hours. The lysate was

centrifuged for 20 minutes at 12,000 ×*g* to remove cell debris. The supernatant was retained and treated with RNAase and DNAase before removing it from the category 3+ laboratory as described in Section 2.5.1.

## 2.6.6 Hydrophobic Interaction Chromatography (HIC)

The HiTrap HIC Test Kit (Pharmacia) was used to screen for the most appropriate HIC medium for the purification of rDNT. The Test Kit consisted of five hydrophobic interaction chromatography media with different hydrophobic characteristics. The five different media were pre-packed in 1 ml HiTrap columns. All columns were washed with 4 ml of 70% (v/v) ethanol to strip and regenerate the media. The columns were then equilibrated with 4 ml of column buffer (50 mM sodium phosphate, 1 M ammonium sulphate, pH 7.0). Partially pure rDNT was dialysed overnight against the column buffer and 0.5 ml of the dialysed sample was loaded onto each of the test columns. The columns were washed with 2 ml of column buffer. The proteins were eluted from the columns using a descending step-wise gradient of 1 M to 0 M ammonium sulphate in 50 mM sodium phosphate with 0.1 M increments. Finally the columns were washed with 4 ml distilled water, followed by 4 ml of 20% (v/v) ethanol. Fractions of 1 ml were collected and assayed for rDNT by SDS-PAGE and EBL cells.

## 2.7 Protein Methods

### 2.7.1 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

The method used for SDS-PAGE analysis of proteins is based on the discontinuous buffer system of Laemmli (1970). Resolving gels consisted of 5% or 8% (w/v) acrylamide (Appendix B), unless otherwise stated, and the stacking gels consisted of 4% (w/v) acrylamide (Appendix B). Large gels were run using the PROTEAN II system (Bio-Rad) at 120 volts for 16 hours, and cooled to 20°C using a thermostatic circulator (LKB). Small gels were run using the Mini-PROTEAN II system (Bio-Rad) at 150 volts. Protein samples were boiled with sample buffer (Appendix B) for 3 minutes. The samples were loaded on the SDS-polyacrylamide gel, and electrophoresed until the bromophenol blue dye reached the bottom of the gel. All

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gels were run with SDS molecular weight markers (MW SDS-6H; Sigma) (Appendix C). The proteins were then visualised with silver stain.

## 2.7.2 Silver Stain

SDS-polyacrylamide gels were silver stained according to the method of Heukeshoven and Dernick (1985). Staining solutions are described in Appendix D. After electrophoresis the gel was immediately placed in Fixing Solution for at least 30 minutes. After fixing, the gel was placed in Incubation Solution for 30 minutes, followed by three washes of 5 minutes duration in distilled water. Then the gel was put into Silver Solution for 20 minutes and then into Developing Solution for 5-10 minutes until the protein bands became dark. When the desired contrast was obtained the gel was put into Stop Solution for 10 minutes and washed several times with distilled water. The gel was preserved by drying it onto Whatman filter paper for 2 hours at 65°C on a gel dryer (Bio-Rad).

## 2.7.3 Estimation of protein concentration

The Pierce BCA Protein Assay Reagent was used to determine protein concentration. A 100 μl aliquot of protein sample was mixed with 2 ml of Working Reagent (fifty parts 1% bicinchoninic acid, 2% $Na_2CO_3.H_2O$, 0.16% $Na_2$ tartrate, 0.4% NaOH and 0.95% $NaHCO_3$, mixed with one part 4% $CuSO_4.5H_2O$) and incubated at 60°C for 30 minutes. The sample was cooled to room temperature, and the absorbance was measured at 562nm. A set of protein standards of known concentration was prepared using bovine serum albumin (BSA), and used to plot a standard curve to determine the protein concentration of the samples.

## 2.8 Biological Assays

### 2.8.1 Embryonic Bovine Lung (EBL) Cell Assay

EBL cells were used to quantitatively assay for cytotoxicity of wild-type DNT and rDNT (Rutter & Luther, 1984). EBL cells lines were maintained by Frances Puttock in the cell culture laboratory. Eighty μl of Eagle's minimum essential medium (EMEM) was added to all the wells of a 96-well, flat bottom polystyrene ELISA plate (Corning). Twenty μl of partially purified DNT was added and mixed to the top wells of the plate. By removing 20 μl from the top well and transferring it to the 2nd well

down, the sample was serially diluted down the wells of the plate. After each dilution the pipette tip was changed. An 80 μl suspension of EBL cells, containing $3 \times 10^5$ cells $ml^{-1}$, was added, without mixing, to each of the wells. The plates were covered and incubated at 37°C in 5% $CO_2$ for 72 hours. All samples were assayed in duplicate and the bottom row of wells contained only EMEM and EBL cells to serve as a control.

After incubation the contents of the wells were removed by inverting and gently patting on a paper towel. The cells were fixed and stained for 1 hour with 30 μl of EBL stain (Appendix D). The stain was washed off with tap water, and the plate was left to dry overnight and examined microscopically for cytotoxicity. Cytotoxic activity was visualised by rounding of the cells with spaces in the monolayer. The cytotoxic activity of a sample was taken to be the highest dilution of sample that was cytotoxic to the EBL cells.

## 2.8.2 Maintenance of Swiss 3T3 cells

All cell manipulation was carried out in a class 2 cabinet under sterile conditions and all solutions warmed to 37°C before use. Cell stocks were stored in liquid nitrogen at $2 \times 10^6$ cells $ml^{-1}$ in Dulbecco's modified Eagles medium (DMEM) (ICN Flow) containing 10% foetal calf serum (FCS) and 10% dimethyl sulphoxide. Stock cells (1 ml) were thawed and added slowly to 10 ml of DMEM containing 10% FCS. The cells were centrifuged at 400 ×g for 10 minutes and washed twice with DMEM/FCS. The cells were plated out in a 260 ml tissue culture flask (Nunc) with 10 ml DMEM/FCS and incubated at 37°C in 5% $CO_2$ for 3-4 days.

The medium was removed from the Swiss 3T3 cells and the cells were washed twice with 5 ml versene (PBS with 50 mM EDTA, pH 8.0) at 37°C. This removed any remaining serum which would inhibit the action of trypsin. The versene was removed and 5 ml of 0.05% trypsin/versene mix was added to the flask. The flask was placed at 37°C in 5% $CO_2$ for 5 minutes until all the cells had been lifted off the flask. An inverted microscope was used to check thoroughly that all cells were detached to avoid the selection of variants with altered adhesion properties. The trypsinised cells were transfered into a sterile universal bottle and the flask was rinsed with versene into the same bottle. The cells were pelleted by centrifugation (400 ×g, 5 min) and resuspended in 1 ml DMEM. An aliquot of this cell suspension was counted using a

counting chamber (Weber Scientific International LTD) inorder to determine the dilution factor required. The cells were plated on to 260 ml tissue culture flasks (Nunc) at $6 \times 10^4$ cells in 10 ml DMEM/FCS. The cells were cultured at 37°C in 5% $CO_2$ for 3-4 days.

The cells were subcultured every 3-4 days to maintain subconfluent cultures. This is critical to avoid the emergence in the cell population of variants that are able to proliferate under crowded conditions and consequently have a relaxed density-dependent inhibition of growth.

### 2.8.3 Preparation of Swiss 3T3 cells for experimental purposes

Swiss 3T3 cells were subcultured as described in Section 2.8.2. After 3-4 days the subconfluent cultures were re-fed with fresh DMEM/FCS. The cells were cultured at 37°C in 5% $CO_2$ for a further 2-3 days,after which they had become confluent. The cells were trypsinised, collected and counted as described in Section 2.8.2.

Those cells to be used in fluorescent staining experiments were seeded onto coverslips in 6-well plates (Nunc) at $4 \times 10^4$ cells/well in 2.5 ml DMEM/FCS. The cells were cultured at 37°C in 5% $CO_2$ for 48 hours. The coverslips were sterilised before use by gamma irradiation.

Those cells to be used in [$^3$H]-thymidine incorporation assays for the measurement of DNA synthesis were plated out in 6-well plates (Nunc) at $10^5$ cells/well with 2.5 ml DMEM/FCS. The cells were cultured at 37°C in 5% $CO_2$ for 6-8 days until the cells were confluent and quiescent.

### 2.8.4 Fluorescent staining of actin in Swiss 3T3 cells

Swiss 3T3 cells were seeded onto coverslips and cultured as described in Section 2.8.3. The subconfluent cultures of Swiss 3T3 cells on coverslips in 6-well plates (Nunc) were washed twice with PBS at 37°C to remove residual serum. Samples to be assayed were added at various concentrations to each well in 2.5 ml DMEM. The cells were cultured for indicated periods at 37°C in 5% $CO_2$.

The cells were fixed in fresh 0.1 M phosphate buffered 2% (w/v) paraformaldehyde fixative for 10 minutes at room temperature. New fixative was prepared before each use. The 2% (w/v) paraformaldehyde fixative was prepared by

mixing together 41.5 ml $NaH_2PO_4$ stock (Appendix D) with 1 ml $CaCl_2$ stock (Appendix D) and 40.5 ml distilled water. Then, 0.54 g glucose was dissolved in the mixture. Meanwhile, 17 ml NaOH stock solution (Appendix D) was gently heated in a water bath to just above 60°C. In the fume hood the NaOH stock was poured onto 2 g of paraformaldehyde and stirred throughly. The two solutions were mixed together and allowed to cool before fixing the cells. If the whole immunofluorescence protocol was not processed on the same day the cells were fixed for 30 minutes. The cells were washed twice with PBS and permeablilised by incubating with microtubuli-stabilising buffer (tbs) (Appendix D) for 5 minutes. The cells were washed three times with PBS and placed in blocking buffer (Appendix D) for 10 minutes. If the cells were processed at a later stage, they were left in blocking buffer overnight at 4°C. After blocking, the cells were washed twice with PBS and incubated with a 1/1000 dilution of rhodamine-phalloidin (stock 1 mg/ml in ethanol) (Sigma) in PBS, for 30 minutes at 37°C in a humidified chamber. The cells were washed twice with PBS and finally washed with distilled water. The coverslips were mounted onto a glass slide in fluoromount-G (Southern Biotechnology Associates Inc., USA), and the edges sealed with nail polish. A fluorescent microscope (Diaplan, Leitz) was used to view the cells.

### 2.8.5 Fluorescent staining of vinculin in Swiss 3T3 cells

Swiss 3T3 cells were seeded onto coverslips and cultured as described in Section 2.8.3. The subconfluent cultures of Swiss 3T3 cells on coverslips were washed twice with PBS at 37°C to remove residual serum. Samples to be assayed were added at various concentrations to each well in 2.5 ml DMEM. The cells were cultured for indicated periods at 37°C in 5% $CO_2$.

The cells were fixed, permeabilised and blocked as described in Section 2.8.4. After blocking, the cells were washed twice with PBS and then incubated with 1/100 diluted mouse monoclonal anti-vinculin (clone VIN-11-5, Sigma) in PBS, for 2 hours at 37°C in a humidified chamber. After 2 washes with PBS, the cells were incubated with 5% (v/v) goat serum (Sigma), diluted in PBS, for 20 minutes at 37°C in a humidified chamber. The cells were washed twice with PBS and incubated with a 1/100 dilution of Fluorescein-isothiocyanate-labelled (FITC) goat anti-mouse IgG (Sigma) for 1 hour at 37°C in a humidified chamber. The cells were washed twice

with PBS and finally washed with distilled water. The coverslips were mounted onto a glass slide in fluoromount-G (Southern Biotechnology Associates Inc., USA) and the edges sealed with nail polish. A fluorescent microscope (Diaplan, Leitz) was used to view the cells.

An FITC labelled donkey anti-mouse IgG was used to reduce background. After incubation with the anti-vinculin monoclonal as described above, the cells were incubated with 5% (v/v) donkey serum in PBS, for 20 minutes at 37°C in a humidified chamber. The cells were washed twice with PBS and incubated with a 1/100 dilution of FITC donkey anti-mouse IgG (Jackson Immuno Research Lab. Inc.) for 1 hour at 37°C in a humidified chamber. Finally the cells were washed and mounted as described above.


### 2.8.6 Stimulation of DNA synthesis in quiescent Swiss 3T3 fibroblast cells

This method is based on that described by Higgins and Rozengurt (1994). Swiss 3T3 cells were cultured in DMEM containing 10% FCS until they were confluent and quiescent. Stimulation of DNA synthesis in these cells was measured by incorporation of tritiated [$^3$H]-thymidine into the acid insoluble fractions of the cells.

The confluent cultures of Swiss 3T3 cell in 6-well plates (Nunc) were washed twice with DMEM at 37°C to remove residual serum. Tritiated thymidine (specific activity = 6.7 Ci mM$^{-1}$; 1 mCi ml$^{-1}$ in aqueous solution, NEN Dupont) was added to 1:1 DMEM/Waymouth medium (ICN Flow) to give a final radioactivity of 1 μCi ml$^{-1}$ and 2.5 ml of this medium was added to each well. Samples to be assayed were added to wells at appropriate dilutions. Each sample was assayed in duplicate or triplicate. To obtain a negative control the cells were cultured with 1:1 DMEM/Waymouth medium only, and for a positive control the cells were cultured with DMEM/FCS. The cells were cultured at 37°C in 5% CO$_2$ for 40 hours to allow [$^3$H]-thymidine incorporation to occur.

After 40 hours the medium was discarded and each well washed twice with cold PBS. The plates were incubated for 20 minutes at 4°C with 1 ml of cold 5% (w/v) trichloroacetic acid (TCA) in each well. Acid treatment induced lysis of the cells and precipitated the acid insoluble fraction of the cells. The TCA was removed and

the wells washed twice with 70% (v/v) ethanol. The cells were solubilised by incubation in 1 ml 0.1 M NaOH, 2% (w/v) $Na_2CO_3$ for 30 minutes at 37°C. A 0.5 ml sample from each well was placed in a scintillation vial and mixed with 5 ml of Optiphase Safe scintillation fluid (LKB). Each sample was counted for 2 minutes on a β-scintillation counter (United Technologies Packard, Tricarb 4660).

Results were used only when the counts from the negative control were less than 5% of those from the positive control. The level of incorporation of [$^3$H]-thymidine into cells induced by addition of DMEM/FCS caused maximum stimulation of DNA synthesis. The values given by the DNT samples were expressed as a percentage of the maximal level of DNA synthesis. All samples were assayed in triplicate and each experiment was repeated at least two or three times.

## 2.8.7 Induction of DNA synthesis by transient exposure to rDNT

Confluent, quiescent cultures of Swiss 3T3 cells were washed twice with PBS and incubated with serum free DMEM at 37°C for various times with or without 10 ng ml$^{-1}$ rDNT. After various incubation times the cells were washed five times with PBS and then incubated for 40 hours in 1:1 DMEM/Waymouth medium containing 1 μCi of [$^3$H]-thymidine per ml. One 6-well plate was washed seven times with PBS and continuously exposed to 10 ng ml$^{-1}$ rDNT throughout the 40 hour incubation. DNA synthesis was measured as described in Section 2.8.6

## 2.8.8 Effect of methylamine on DNA synthesis induced by rDNT

Confluent, quiescent cultures of Swiss 3T3 cells were washed twice with PBS and incubated with 1:1 DMEM/Waymouth medium containing 1 μCi of [$^3$H]-thymidine per ml, with various concentrations of methylamine hydrochloride (pH 7.0) at 37°C for 1 hour. Then, 10 ng ml$^{-1}$ of rDNT or 10 ng ml$^{-1}$ PMT was added to the cells. The Swiss 3T3 cells were incubated in this medium for 40 hours, and DNA synthesis was measured as described in Section 2.8.6.

Confluent, quiescent cultures of Swiss 3T3 cells were washed twice with PBS and incubated with 1:1 DMEM/Waymouth medium containing 1 μCi of [$^3$H]-thymidine per ml, with 10 ng ml$^{-1}$ rDNT. At various times from 0 to 6 hours, methylamine hydrochloride (pH 7.0) was added to the cultures to give a final

concentration of 7 mM. DNA synthesis was measured after 40 hours as described in Section 2.8.6.

### 2.8.9 Measurement of polynucleated cell number

Swiss 3T3 cells were seeded onto coverslips and cultured as described in Section 2.8.3. The subconfluent cultures of Swiss 3T3 cells on coverslips in 6-well plates (Nunc) were washed twice with PBS at 37°C to remove residue serum. Samples to be assayed were added at various concentrations to each well in 2.5 ml DMEM. The cells were cultured for 24 hours at 37°C in 5% $CO_2$.

The medium was removed from the plates and the cells fixed for 5 minutes in a mixture of acetic acid/ methanol (1:3). The fixative was removed and fresh fixative added for a further 5 minutes. The cells were air dried, and then incubated with Hoechst stain (2.5 μg ml) (Sigma) for 30 minutes in the dark. The cells were washed twice with distilled water and mounted onto slides.

The number of mono-, bi- and poly-nucleated cells in four fields were counted under fluorescence and phase contrast microscopes. The number of binucleated cells in the four fields was divided by the total number of cells in the same fields, and a percentage of binucleated cells was obtained.

### 2.8.10 Cell Number

Swiss 3T3 cells were seeded at 30,000 cells/well in 2.5 ml DMEM/FCS in a 6-well plate (Nunc). The cells were cultured at 37°C in 5% $CO_2$ for 48 hours. The subconfluent cultures of Swiss 3T3 cells were washed twice with PBS at 37°C to remove residual serum. Recombinant DNT was added at a final concentration of 2.5 ng ml$^{-1}$ to each well in 2.5 ml DMEM. As a negative control the cells were cultured with DMEM only, and for the positive control the cells were cultured with DMEM/FCS. The cells were cultured for various times at 37°C in 5% $CO_2$.

Every 24 hours the cells were counted from two wells treated with rDNT and two wells from the positive and negative controls. The medium was removed and the cells washed twice with 2.5 ml versene (PBS with 50 mM EDTA, pH 8.0) at 37°C. The versene was removed and 1 ml of 0.05% trypsin/versene mix was added to the wells. The multi well plate was placed at 37°C in 5% $CO_2$ for 5 minutes until all the

cells had been lifted off the wells, as checked using an inverted microscope. Care was taken that all cells were detached, especially cells treated with rDNT as they were attached strongly to the wells and required a longer incubation period in the trypsin/versene mix. The trypsinised cells were removed into separate sterile universal bottles and the wells were rinsed with versene into the same bottles. The cells were pelleted by centrifugation (400 ×g, 5 min) and resuspended in 1 ml PBS. Each cell suspension was counted four times using a counter chamber (Weber Scientific International LTD).

## 2.8.11 Modification of Rho by rDNT in vivo

Swiss 3T3 cells were seeded at $10^5$ cells/well in 2.5 ml DMEM/FCS in a 6-well plate (Nunc). The cells were cultured at 37°C in 5% $CO_2$ for 6 days. The confluent cultures of Swiss 3T3 cells were washed twice with PBS at 37°C to remove residual serum. Recombinant DNT was added at a final concentration of 10 ng ml$^{-1}$ to each well in 2.5 ml DMEM. Control cells were cultured with DMEM only, or DMEM/FCS. The cells were cultured for 24 hours at 37°C in 5% $CO_2$.

After 24 hours the medium was removed and the cells washed twice with 2.5 ml versene (PBS with 50 mM EDTA, pH 8.0) at 37°C. The versene was removed and 1 ml of 0.05% trypsin/versene mix was added to the wells. The multi well plate was placed at 37°C in 5% $CO_2$ for 5 minutes until all the cells had been lifted off the wells, as checked using an inverted microscope. Proceedures to transfer all cells into universal bottles are described in Section 2.8.10. The cells were pelleted by centrifugation (400 ×g, 5 min) and resuspended in 1 ml PBS. Each cell suspension was counted using a counting chamber (Weber Scientific International LTD). The cells were pelleted by centrifugation (400 ×g, 5 min) and resuspended in NP40 lysis buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.5% NP40) to give a final concentration of $10^7$ cells/ml. The cell suspensions were left at room temperature for 10 minutes. The lysates were centrifuged for 5 minutes at 400 ×g to remove cell debris. The supernatants were retained and analysed by Western blot.

The cell lysates from rDNT-treated Swiss 3T3 cells and control cells, and purified recombinant Rho were separated by 15% SDS-PAGE using the technique described in Section 2.7.1. The samples were blotted onto Hybond nitrocellulose

membrane (Amersham) in transfer buffer (25 mM Tris, 192 mM glycine, 25% (v/v) methanol) at 30 volts overnight using the technique described for electroblotting in Section 2.9.6.

After electroblotting, the membrane was blocked for 3 hours in 5% (w/v) skimed milk (Marvel) in PBS, and then washed three times in PBS; each wash was for 10 minutes. Then the membrane was probed with 1/100 dilution of anti-RhoA monoclonal antibody (26C4) (Santa Cruz Biotechnology, USA) in 1% (w/v) Marvel/PBS, for 2 hours at room temperature. The membrane was washed three times for 10 minutes in PBS, and then probed with 1/1000 dilution of rabbit anti-mouse immunoglobulin-HRP (DAKCO) in 1% (w/v) Marvel/PBS, for 2 hours at room temperature. The membrane was washed three times in PBS for 10 minutes, and developed using ECL detection reagents (Amersham) as described in Section 2.9.6.

## 2.9 Immunological methods

### 2.9.1 Detoxification of DNT

Partially purified DNT from *B. bronchiseptica* B58 was detoxified by dialysing against 1 litre of 1% (v/v) formaldehyde in PBS at room temperature for 24 hours. Then the sample was dialysed against several portions of fresh PBS for 24 hours. Detoxified DNT was analysed for remaining cytotoxic activity using the EBL cell assay.

### 2.9.2 Immunisation procedure

Mouse immunisation was carried out by B. Jones. Four Balb/c mice were injected subcutaneously with 200 µl of a 1:1 dilution of a sample containing toxoid DNT (final protein concentration 15 µg ml$^{-1}$) and Freund's incomplete adjuvant (Sigma). A subsequent injection was given two weeks later, and one week after this injection a serum sample was obtained from each mouse.

The antibody titre of the test serum was determined by ELISA, and the mouse with the highest titre was selected for monoclonal antibody production. The serum was also tested on Western blots against partially purified DNT to show whether the serum contained antibodies against DNT.

## 2.9.3 ELISA for the measurement of serum antibody response

Partially purified DNT was diluted in 50 mM carbonate-bicarbonate buffer, pH 9.2, to give a total protein concentration range from 3 $\mu g\ ml^{-1}$ to 0.5 $\mu g\ ml^{-1}$. A 50 $\mu l$ volume of each dilution was added to each well of a single column of a 96 well 3912 microtest III flexible assay plate (Falcon). The plate was incubated overnight at 4°C then washed 3 times with PBS containing 0.05% (v/v) Tween 20, and then blotted dry. Fifty $\mu l$ of 3% (w/v) BSA in PBS was added to each well, and the plate was left at room temperature for 2 hours and washed as above.

Fifty $\mu l$ of PBS was dispensed into each well of the plate; then 50 $\mu l$ of 1/100 dilution of the pooled mouse test serum in PBS was added to the top well. Two-fold serial dilutions were made down the plate to give a final dilution of 1/25600 of the undiluted pooled serum. The plate was then incubated at room temperature for 1 hour before washing as above.

Rabbit anti-mouse immunoglobulin, conjugated to horseradish peroxidase (RAM-HRP) (DAKCO), was diluted to 1/1000 in PBS and 50 $\mu l$ was added to each well. The plate was incubated at room temperature for 1 hour before washing as above.

The titre of antiserum was detected colourimetrically by adding 50 $\mu l$ of tetramethylbenzamine substrate to each well. After 10 minutes a blue colour developed in the positive wells. The reaction was stopped by adding 12.5 $\mu l$ of 2 M sulphuric acid to each well. The intensity of the yellow colour reaction was measured at 450nm on a multiskan MCC spectrophotometer (Titertek).

The above method was repeated using various blocking methods. The plates were blocked with 3% (w/v) BSA, 5% (w/v) BSA, 20% (w/v) Marvel, or 1% (w/v) gelatin for 2 hours before the pooled serum was added to the wells. The pooled serum and RAM-HRP were diluted in 1% (w/v) BSA or 2% (w/v) Marvel in certain screens.

The appropriate parameters for the ELISA were determined. A titration using serial dilutions of individual immune mouse sera was carried out as above using a single coating antigen concentration (1 $\mu g\ ml^{-1}$ protein). The plate was blocked with 20% (w/v) Marvel in PBS, and the sera and RAM-HRP were diluted to 1/1000 in 2% (w/v) Marvel in PBS. The antibody response of the four mice was compared with the pooled antiserum preparation and the titre of normal mouse serum.

## 2.9.4 Cell fusion

An intraperitoneal injection of toxoid DNT (12.5 μg protein in 200μl PBS) was given to mice numbers two and four, 12 weeks after the sample serum was taken.

Hybridomas were prepared by Brenda Jones using the standard methods as described by Campbell (1991).

Samples (100 μl) were removed from hybridoma colonies and tested by ELISA, to establish which hybridomas secreted antibody molecules that recognised antigen from partially purified *B. bronchiseptica* DNT samples. Each of the colonies from wells giving positive results was transferred to fresh cluster plates. Five days later, the supernatant fluids from these hybridomas were screened by Western blot. The hybridomas which recognised DNT by Western blot were frozen in the gaseous phase over nitrogen.

## 2.9.5 Protein G Sepharose purification of monoclonal antibodies

Culture fluid supernatant from monoclonal hybridomas was filtered through a 0.45 μm pore-size membrane and loaded onto a protein G Sepharose column (5 ml) (Pharmacia) equilibrated with 20 mM sodium phosphate, pH 8. The column was washed with 50 ml of 20 mM sodium phosphate, pH 8. The last 10 ml of the wash was collected as 2 ml fractions for spectrophotometric readings at 280nm. The bound antibody was eluted with 30 ml of 0.1 M glycine, pH 2.7 and collected as 1 ml fractions in tubes containing 60 μl of 1 M Tris/HCL, pH 9, to neutralise the elutant. The column was stripped with 20 ml of 6 M guanidine hydrochloride and regenerated by washing with 50 ml of 20 mM sodium phosphate, pH 8. Protein G Sepharose was stored at 4°C in 20% (v/v) ethanol.

The O.D. of the fractions collected were read at 280nm, and the antibody was usually eluted from the column at approximately fraction 6 after addition of elution buffer. The antibody containing fractions were pooled and dialysed overnight at 4°C against PBS. The antibody was divided into aliquots and stored at -20°C.

## 2.9.6 Immunoblotting

A SDS-polyacrylamide gel was run as described in Section 2.7.1 using the mini-PROTEAN II system (Bio-Rad). Pre-stained molecular weight markers (Appendix C) were used to provide a visual check of transfer efficiency. The gel was then electroblotted onto ProBlot membrane (Applied Biosystems). The gel was assembled into the transblotting sandwich, which consisted of, in order, a fibrous pad, two sheets of filter paper, the gel, the ProBlot membrane, two sheets of filter paper and a fibrous pad. The CAPS transfer buffer was prepared by mixing 200 ml of CAPS stock solution (Appendix E) with 200 ml methanol and 1,600 ml distilled water. All components were pre-equilibrated in CAPS transfer buffer. The transblotting sandwich was put into the mini-PROTEAN II transblotting apparatus (Bio-Rad) with the ProBlot membrane nearest the anode. The protein was electroblotted for 6 hours at 30 volts.

After electroblotting the membrane was stained for 1-2 minutes with Ponceau S (Appendix D) to visualise the transferred proteins. The membrane was washed several times with distilled water to remove the stain, and blocked overnight at 4°C in 5% (w/v) Marvel in PBS. The membrane was washed three times in PBS; each wash was for 10 minutes. Then, the membrane was probed with a 1/100 dilution of anti-DNT monoclonal antibody in 1% (w/v) Marvel/PBS, for 2 hours at room temperature. The membrane was washed three times for 10 minutes in PBS, and then probed with a 1/1000 dilution of rabbit anti-mouse immunoglobulin-HRP (DAKCO) in 1% (w/v) Marvel/PBS, for 2 hours at room temperature. The membrane was washed three times in PBS for 10 minutes, and developed using ECL detection reagents (Amersham). This allows detection by chemiluminescence of immobilised antigens, conjugated directly or indirectly with HRP-conjugated antibodies. Equal volumes of ECL detection solution 1 was mixed with detection solution 2, and then the membrane was incubated in the detection reagent for 1 minute. Excess reagent was drained off and the membrane wrapped in clingfilm, smoothing out air pockets. The membrane was placed, protein side up, in a film cassette. In the dark, a sheet of autoradiography film (Kodak) was placed on top of the membrane, the cassette was closed and exposed for between 30 seconds and 5 minutes and the film was developed.

## 2.9.7 Neutralisation of cytotoxic activity of DNT on EBL cells by monoclonal antibodies

Partially pure DNT was diluted in EMEM in a microtitre plate as described in Section 2.8.1. To each well was added 20 µl of a 1/100 dilution of purified monoclonal antibody diluted in PBS. The microtitre plate was incubated for 1 hour at 37°C in 5% $CO_2$ to allow the binding of antibody to toxin. To each well 100 µl of EBL cell suspension (3 x $10^5$ cells $ml^{-1}$) was added, and the cells were incubated and stained as described in Section 2.8.1.

## 2.9.8 Biotinylation of monoclonal antibodies

Monoclonal antibodies were labelled with biotin for use in competition ELISA. Monoclonal antibody was prepared at 1 mg $ml^{-1}$ in 0.1 M sodium phosphate buffer, pH 7.2. Biotinamidocaproate-N-hydroxy-sulfosuccinimide ester (BAC-SulfoNHS) (Sigma) was dissolved at 5 mg in 30 µl DMSO. The dissolved BAC-SulfoNHS was made up to 0.5 ml with 0.1 M sodium phosphate buffer, pH 7.2, and vortexed well. The resulting concentration of the BAC-SulfoNHS solution was 10 mg $ml^{-1}$. Immediately after preparation 3.7 µl of the BAC-SulfoNHS solution was added to 1 ml of antibody to give a 10:1 molar ratio and incubated at room temperature for 30 minutes with shaking.

The unbound biotin was removed by centrifugation in a microcon 30 (Amicon). The antibody and BAC-SulfoNHS mixture was centrifuged for 10-15 minutes at 1000 rpm. After centrifugation 100 µl of 0.1 M sodium phosphate buffer was added to the mixture and recentrifuged. This process was repeated twice.

The monoclonal antibodies were tested for biotinylation by Western blot. Western blots of partially pure DNT and rDNT were probed with a 1/100 dilution of biotinylated anti-DNT monoclonal antibody in 1% (w/v) Marvel/PBS, for 2 hours at room temperature. The membrane was washed three times for 10 minutes in PBS, and then probed with 1/1000 dilution of strepavidin-HRP (Amersham) in 1% (w/v) Marvel/PBS, for 2 hours at room temperature. The membrane was washed three times in PBS for 10 minutes, and developed using ECL detection reagents (Amersham) as described in Section 2.9.8. The biotinylated monoclonal antibodies were stored at 4°C.

## 2.9.9 Competition ELISA

Partially purified DNT was diluted in 50 mM carbonate-bicarbonate buffer, pH 9.2, to give a total protein concentration 1 µg ml$^{-1}$. A 50 µl volume of diluted DNT was added to each well of a 96 well 3912 microtest III flexible assay plate (Falcon). The plate was incubated overnight at 4°C, then washed 3 times with PBS containing 0.05% (v/v) Tween 20, and then blotted dry. Into each well 50 µl of 20% (w/v) Marvel in PBS was added, and the plate was left at room temperature for 2 hours and washed as above.

Fifty µl of 2% (w/v) Marvel/PBS was dispensed into each well of the plate, and then 50 µl of a 1/100 dilution of the biotinylated anti-DNT monoclonal antibody in PBS was added to the top well. Two-fold serial dilutions were made down the plate. Fifty µl of a 1/100 dilution of the unlabelled, competing anti-DNT monoclonal antibody in PBS was added to wells of column 8. Two-fold serial dilutions were made across the plate. Controls included: i) a serial dilution of biotinylated anti-DNT monoclonal antibody only, and ii) the unlabelled, competing anti-DNT monoclonal antibody only. The plate was then incubated at room temperature for 1 hour before washing as above.

Strepavidin conjugated to horseradish peroxidase (Amersham) was diluted to 1/1000 in 2% (w/v) Marvel/PBS and 50 µl was added to each well. The plate was incubated at room temperature for 1 hour before it was washed as above.

The titre of antiserum was detected as described in Section 2.9.3.

# *Chapter 3*
# *Purification of Bordetella bronchiseptica*
# *dermonecrotic toxin*

## 3.1 Introduction

It is important to obtain pure protein in order to be able to study its activity, structure or its structural-functional relationship. All proteins have unique chemical and physical properties, such as size, charge, solubility, and specific binding affinities, which can be exploited to separate a chosen protein from a mixture of proteins. Protein purification methods are based on: i) charge (e.g. ion-exchange chromatography, isoelectric focusing); ii) hydrophobicity (e.g. hydrophobic interaction chromatography); iii) size (e.g. gel filtration, dialysis); iv) activity (e.g. affinity chromatography, immunopurification). When planning a purification strategy each technique should be evaluated for its capacity, resolving power, probable protein yield and cost.

Despite the fact that DNT was first reported in 1909 by Bordet and Gengou, it was not until 1969 that DNT was purified by Nakase et al. (1969) from B. pertussis, and 1985 when Nakase and Endoh purified DNT from B. bronchiseptica. Since 1985 several methods for the purification of DNT have been published using various purification techniques (see Table 3.1). The purification of DNT has been hindered by protein instability, and its poor and unreliable recovery (Wardlaw and Parton, 1983). DNT is heat-labile, for example it is inactivated when heated to 56°C for 30 minutes (Munzo, 1971). Crude preparations of DNT stored at 37°C lost activity within days (Livey and Wardlaw, 1984), whereas, Zhang and Sekura (1991) reported that purified DNT lost half of its activity when stored at 37°C for just 75 minutes. There is considerable variation in the results on the stability of DNT stored at 4°C. For example, crude preparations of B. pertussis DNT lost 50% of their activity after 1 week at 4°C (Munzo, 1971), whereas purified DNT from B. bronchiseptica was stable for 3 months at 4°C in 50 mM phosphate buffer, pH 7.1, containing 0.3 M sodium sulphate and 1 M urea (Horiguchi et al., 1990). The findings of Garrod (1994) and Livey and Wardlaw (1984) that partially pure DNT was more stable at -20°C than at 4°C, are contary to the results of Horiguchi et al. (1990) and Zhang and Sekura (1991).

DNT is considered difficult to purify because it tends to aggregate (Horiguchi et al., 1989) and form complexes with other co-existing substances such as lipids, acidic proteins and hydrophobic substances (Endoh et al., 1986c). It was also reported

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to be insoluble at low ionic strength (Endoh *et al.*, 1986c). Horiguchi *et al.* (1989) found that DNT co-purified with a non-toxic and immunologically distinct 91 kDa protein, which could be separated by ion-exchange chromatography in the presence of 1 M urea. Zhang and Sekura (1985; 1991) reported that DNT co-purified with adenylate cyclase activity, which was resolved by ion-exchange and hydroxylapatite chromatography. In addition DNT has been shown to purify with sugars (Nakase *et al.*, 1969; Kume *et al.*, 1986).

Various methods have been used to increase the stability of DNT and prevent its aggregation to other substances. Most of the purification methods were performed at 4°C to prevent loss of the toxin by heat inactivation (Livey and Wardlaw, 1984; Endoh *et al.*, 1986c; Kume *et al.*, 1986; Horiguchi *et al.*, 1989; Zhang and Sekura, 1991; Garrod, 1994). Protease inhibitors such as phenylmethylsulphonyl fluoride, soyabean trypsin inhibitor, and benzamide, were included in buffers during the purification of DNT from *B. pertussis* to prevent loss of activity (Zhang and Sekura, 1991). Long purification procedures are undesirable due to the instability of the toxin, but some of the published methods take up to 2 weeks to complete (Horiguchi *et al.*, 1989), and some have over ten different purification steps (Endoh *et al.*, 1986c; Kume *et al.*, 1986). Methods that may cause aggregation of the toxin, such as dialysis and ultrafiltration, should also be avoided. Horiguchi *et al.* (1989) used 1 M urea in the final buffer to avoid aggregation of DNT, and found that urea was essential for the stability of the toxin (Horiguchi *et al.*, 1990).

Properties of the purified DNT vary widely (see Table 3.1), probably due to the use of different purification approaches. Furthermore the molecular weight of purified DNTs varied between 89 kDa (Livey and Wardlaw, 1984) and 190 kDa (Kume *et al.*, 1986), but this variation may be an artifact of the different techniques used to determine the molecular weight of DNT. Molecular weight was determined by gel filtration (Livey and Wardlaw, 1984; Endoh *et al.*, 1986c), SDS-PAGE (Zhang and Sekura, 1985; Kume *et al.*, 1986; Horiguchi *et al.*, 1989) or both (Horiguchi *et al.*, 1990; Zhang and Sekura, 1991). Additionally, the variation in molecular weight may be due to co-purified proteins in preparations thought to be pure DNT. The potent biological activity of DNT may have been attributed to other proteins in preparations where the low level of DNT was not detected by standard protein detection

procedures.

Most published methods show that DNT is a single polypeptide chain. In contrast to this, *B. bronchiseptica* DNT, purified by Endoh *et al.* (1986c), was reported to be a tetramer of 102 kDa, consisting of two distinct subunits of 30 and 20 kDa. However, when DNT was purified from the same strain using the purification method of Horiguchi *et al.* (1990), it was reported to be a single polypeptide chain of 145 kDa. There are also contradictory reports on the possible secondary and tertiary structure of DNT. Mildly trypsinised DNT purified by Kume *et al.* (1986) dissociated into two polypeptide chains, with molecular weights of 75 and 118 kDa, after treatment with dithiothreitol-SDS or urea. When the dithiothreitol and urea were removed from the dissociated DNT, the fragments reassociated, and the DNT formed was indistinguishable from the native toxin. Kume *et al.* (1986) concluded that trypsin cleaves DNT into two fragments which are linked by one or more disulphide bonds. Endoh *et al.* (1986c) suggested that DNT contains no disulphide bonds, because purified DNT was unaffected by disulphide bond-reducing agents, and because cysteine was not detected in the toxin by amino acid analysis. However, the dervied amino acid sequence for *B. bronchiseptica* DNT contains eight cysteine residues (Pullinger *et al.*, 1996) and *B. pertussis* DNT contains nine cysteine residues (Walker and Weiss, 1994).

None of the methods published so far provides good evidence that the purified DNT is truly homogeneous. No single method has become universally accepted as a reproducible method of purification. In this laboratory Garrod (1994) developed a simple purification procedure to produce partially pure *B. bronchiseptica* DNT by sonication, ion-exchange and affinity chromatography. Ion-exchange chromatography separates proteins on the basis of their charge. DEAE-Sephacel is an anion exchanger used in the purification of negatively charged proteins. The column buffer was at pH 7.5 to keep DNT (pI approx. 6.5) negatively charged. Hydroxylapatite chromatography is an affinity technique in which protein adsorption involves both $Ca^+$ and $PO_4^{3-}$ groups on a crystalline surface which bind to acidic and basic proteins respectively. This purification method was proven to be quick, reliable and reproducible in yielding partially pure DNT. These preparations of DNT were cytotoxic to EBL cells, and contained a protein of 145 kDa as determined by SDS-PAGE. A protein of 155 kDa

and several proteins about 45 kDa and less co-purified with DNT. One aim of my research was to obtain DNT of greater purity by employing both published and new purification methods.

Horiguchi *et al.* (1989; 1990) used Kurimover C-7, a water soluble chitin derivative, to remove nucleic acids and acidic proteins from sonicated cell extracts in the purification of *B. bronchiseptica* DNT. They reported that nucleic acids affect ion exchange chromatography, and that dermonecrotic activity of DNT was seen only when Kurimover C-7 was used (Horiguchi *et al.*, 1990). Zhang and Sekura (1991) used ammonium sulphate fractionation in the purification of *B. pertussis* DNT which removes nucleic acids from the sonicated cell extract. The effect of Kurimover C-7 treatment on *B. bronchiseptica* sonicated cell extracts was investigated to see if it improved the purification of DNT.

Horiguchi *et al.* (1989) used 1 M urea in the ion exchange chromatography buffer during the final stages of the purification of DNT, because urea was shown to prevent aggregation of the toxin without affecting its dermonecrotic activity. In a simpler purification procedure for *B. bronchiseptica* DNT, 1 M urea was used in the buffer throughout the purification (Horiguchi *et al.*, 1990). Urea appeared to be essential for DNT stability, and pure DNT was stable for 3 months at 4°C, when stored in 50 mM phosphate buffer, pH 7.1, containing 1 M urea and 0.3 M $Na_2SO_4$.

Partially purified DNT from our laboratory was only stable for about 3-4 weeks at 4°C. Many methods, such as gel filtration and dialysis, have been investigated to purify DNT further, but have failed due to apparent aggregation of DNT to cellular macromolecules or dialysis tubing (Garrod, 1994). The effect of urea on purification was investigated to see if it would prevent aggregation and increase the stability of the toxin.

Finally, a new approach was adopted to separate DNT from the other cell components on the basis of its isoelectric point (pI). Proteins are amphoteric molecules with a pH dependant net charge. Their overall charge is determined by the ionisable acidic and basic side chains of the amino acids. Carboxylic acid groups (-COOH) are uncharged in acidic solutions and are negatively charged at higher pH values (-COO⁻). Amine groups are uncharged at alkaline pH (-$NH_2$), but are positively charged at lower pH values (-$NH_3^+$). The specific pH value in which the net charge of a protein is zero

| | | | | | |
|---|---|---|---|---|---|
| | | 3) gel filtration<br>4) ion-exchange<br>5) hydroxylapatite<br>6) HPLC hydroxylapatite | | | |
| Endoh *et al.*, 1986c | *B. bronchiseptica* | 1) sonication<br>2) hydrophobic interaction<br>3) ultrafiltration<br>4) sucrose density gradient centrifugation<br>5) ultracentrifugation<br>6) gel filtration<br>7) ultrafiltration<br>8) gel filtration<br>9) ultrafiltration<br>10) isoelectric precipitation<br>11) ultrafiltration<br>12) isoelectric focusing<br>13) ultrafiltration<br>14) isoelectric focusing<br>15) ultrafiltration | 102 | 6.9 | |
| Kume *et al.*, 1986 | *B. bronchiseptica* | 1) sonication<br>2) ion-exchange<br>3) ultrafiltration and dialysis<br>4) ion-exchange<br>5) ultrafiltration and dialysis<br>6) gel filtration<br>7) lyophilization<br>8) dialysis<br>9) preparative PAGE<br>10) acid precipitation<br>11) gel filtration<br>12) lyophilization | 190 | 6.5-6.6 | |
| Horiguchi *et al.*, 1989 | *B. bronchiseptica* | 1) sonication<br>2) DNase and RNase<br>3) ion-exchange<br>4) hydroxylapatite<br>5) ultrafiltration<br>6) gel filtration<br>7) hydroxylapatite<br>8) dialysis<br>9) ion-exchange | 145 | 6.3-6.7 | |
| Horiguchi *et al.*, 1990 | *B. bronchiseptica* | 1) sonication<br>2) DNase and RNase<br>3) dialysis<br>4) ion-exchange<br>5) ultrafiltration<br>6) HPLC gel filtration | 145 | | |
| Zhang and Sekura, 1991 | *B. pertussis* | 1) sonication<br>2) ammonium sulphate precipitation<br>3) affinity chromatography<br>4) gel filtration<br>5) ion-exchange<br>6) hydroxylapatite | 140 | 6.02 | |
| Garrod, 1994 | *B. bronchiseptica* | 1) sonication<br>2) ion-exchange<br>3) hydroxylapatite | 145 | ND | |

**Table 3.1** Summary of published *Bordetella* DNT purification methods and properties of purified DNTs. Adapted from Garrod (19
ND = not determined
LD$_{50}$ = dose of toxin that killed 50% of inoculated mice in a given time

is known as the protein's isoelectric point (pI). During electrophoresis through an established pH gradient, a protein will migrate until it reaches the pH where its net charge is zero, at this point the protein will stop migrating and will be focused.

There is variation in the reported pI of DNT purified by the different research groups. *B. bronchiseptica* DNT purified by Endoh *et al.* (1986c) had a pI 6.9, whilst Kume *et al.* (1986) purified DNT with a pI 6.5-6.6. DNT purified by Horiguchi *et al.* (1989) was reported to have heterogenous charge, as it focused over a wide range, from pH 6.3 to 6.7.

In our laboratory 2D PAGE of partially purified DNT indicates that DNT has a pI of about 6.5 (P. Ward, personal communication). Preparative isoelectric focusing (IEF) was used to attempt to purify DNT from other cell components. For the preparative IEF, I used the Rotofor cell (Bio-Rad) a preparative scale, free solution, IEF apparatus, originally described by Egen *et al.* (1983).

## 3.2 Results

### 3.2.1 Removal of nucleic acids from B. bronchiseptica sonicated cell extracts

The purification procedure of Garrod (1994) was repeatable, and partially pure DNT was obtained from cell sonicate extracts of *B. bronchiseptica* B58 by DEAE-Sephacel (Figure 3.1) and hydroxylapatite chromatography (Figure 3.2). To investigate if this purification procedure was improved by the removal of nucleic acids, sonicate cell extract from *B. bronchiseptica* was treated with Kurimover C-7. *B. bronchiseptica* sonicate extract treated with Kurimover C-7 showed a 17% decrease in nucleic acids and a 33% decrease in total protein, compared to before the addition of Kurimover C-7 (Table 3.2). The cytotoxic activity of the sonicated cell extract on EBL cells decreased by 80% after Kurimover C-7 treatment. SDS-PAGE analysis of proteins precipitated by Kurimover C-7 showed a protein band of approximately 145 kDa, equivalent to that of DNT (data not shown).

The sonicated cell extract treated with Kurimover C-7 was purified by DEAE-Sephacel chromatography under exactly the same conditions as those described for untreated extracts. The DEAE-Sephacel elution profile was similar whether or not the sonicated cell extract was pre-treated with Kurimover C-7. But with Kurimover C-7 treatment the 145 kDa toxin band was much fainter. DNT-containing fractions from the chromatography of the sonicated cell extract treated with Kurimover C-7 were cytotoxic on EBL cells. However, these fractions were not diluted to an end point in the EBL cell assay. Therefore, it is not possible to deduce whether fractions containing the toxin were less cytotoxic than similar but untreated fractions. Kurimover C-7 treatment of sonicated cell extract did not improve the purification of DNT.

### 3.2.2 Effect of urea on dialysis of DNT

The dialysis of partially purified DNT against 50 mM potassium phosphate, pH 7.1, did not result in the loss of DNT as reported by Garrod (1994). The cytotoxic activity of DNT on EBL cells was the same before and after dialysis (Table 3.3). Dialysis resulted in a very slight loss of protein (Table 3.3), but the protein profile by SDS-PAGE was the same before and after dialysis (data not shown). The dialysis of DNT against 50 mM potassium phosphate, pH 7.1, containing 1 M urea did not affect the cytotoxicity of DNT on EBL cells, nor the concentration of protein (Table 3.3).

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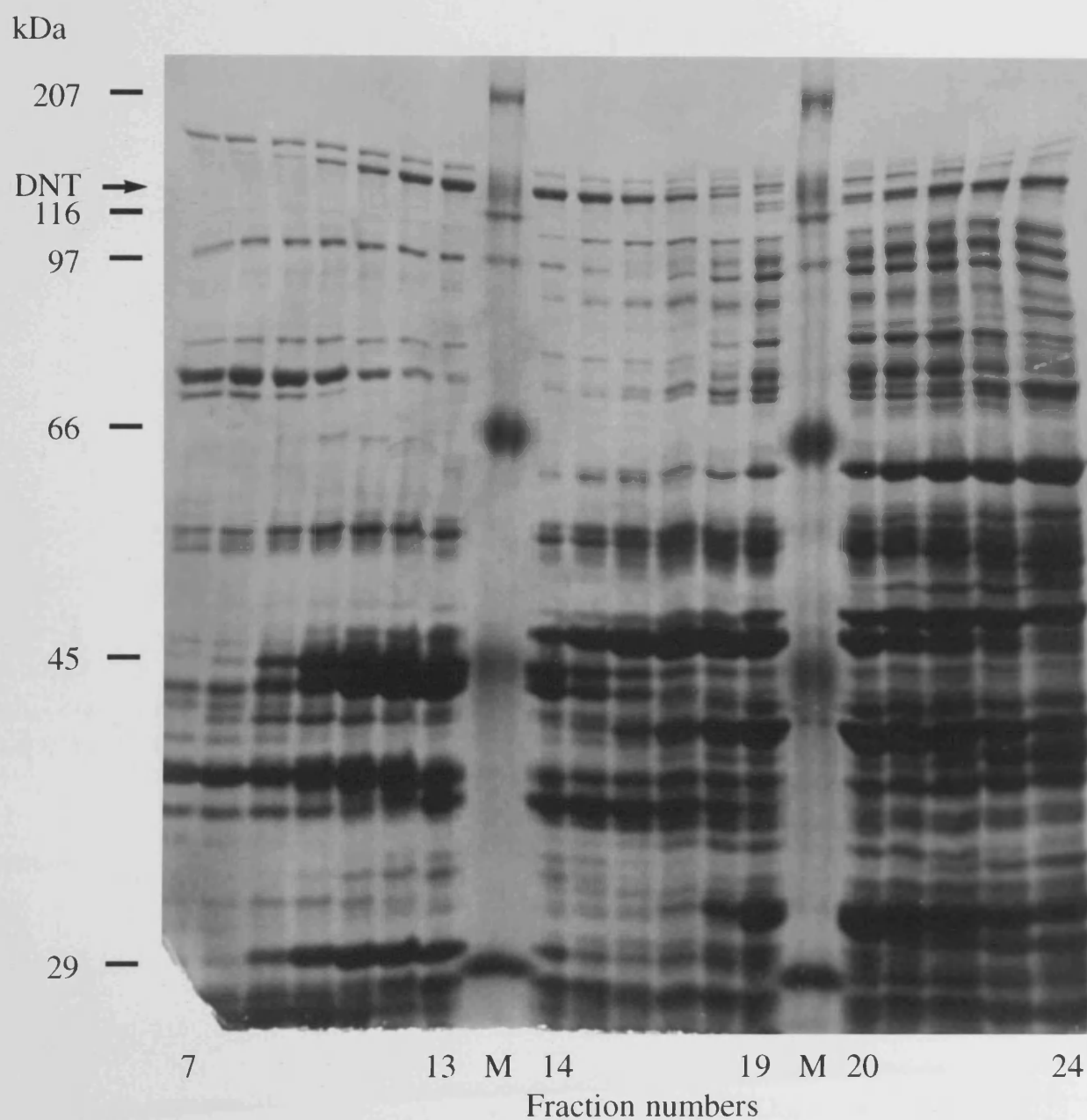
**Figure 3.1** Silver stained 8% SDS-PAGE of DEAE-Sephacel column fractions collected during purification of DNT from *B. bronchiseptica* B58. All lanes contain the same volume of sample.
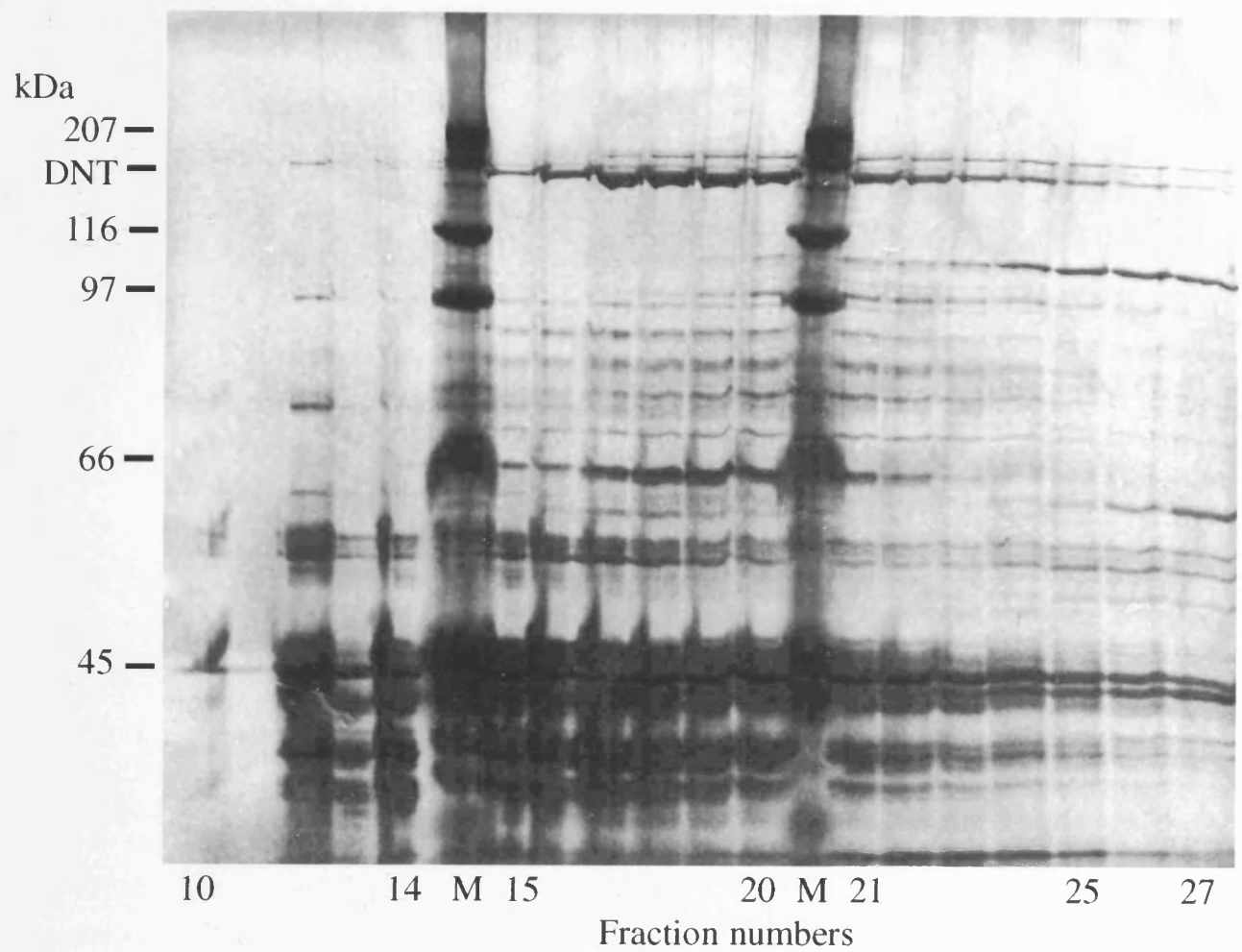
**Figure 3.2** Silver stained 8% SDS-PAGE of hydroxlapatite chromatography fractions collected during the purification of DNT from *B. bronchiseptica* B58.
All lanes contain the same volume of sample.

| Kurimover C-7 treatment | Total protein (mg) | Total nucleic acid (mg) | Cytotoxic activity 1/dilution |
|---|---|---|---|
| Before | 120 | 17.08 | 9,765,625 |
| After | 80 | 14.4 | 1,953,125 |

**Table 3.2** The effect of Kurimover C-7 on the total protein and nucleic acid content, and the cytotoxic activity of sonicate extract of *B. bronchiseptica*.

| Partially pure DNT dialysed against | Protein concentration ($\mu$g/ml) | Cytotoxicity activity 1/dilution |
|---|---|---|
| Before Dialysis | 140 | 9,765,625 |
| No Dialysis | 145 | 9,765,625 |
| 50 mM $K_2PO_4$, pH 7.1 | 125 | 9,765,625 |
| 50 mM $K_2PO_4$, pH 7.1 + 1M urea | 130 | 9,765,625 |

**Table 3.3** The effect of dialysis against 50 mM $K_2PO_4$, pH 7.1, with or without 1 M urea, on the protein concentration and cytotoxic activity of partially pure DNT.

### 3.2.3 Effect of urea and sodium sulphate on gel filtration of partially purified DNT

This work was performed in collaboration with Jim Sinnet-Smith at ICRF, London. Superose 12 gel filtration columns have a fractionation range of 1-300 kDa. Previous attempts to purify DNT on Superose 12 were unsuccessful either due to aggregation of the toxin to other proteins or alternatively aggregation to the column matrix, resulting in DNT being eluted later than expected in fractions containing low molecular weight proteins (Garrod, 1994).

Gel filtration was repeated with 1 M urea and 0.3 M $Na_2SO_4$ in the column buffer. A concentrated sample of partially pure DNT in 50 mM phosphate buffer, pH 7.1, containing 1 M urea and 0.3 M $Na_2SO_4$ was loaded onto the column and eluted under pressure. Analysis of the fractions eluted from the gel filtration column by SDS-PAGE showed a protein band of 145 kDa in fractions 13 and 14 (Figure 3.3). These fractions also contained proteins of lower molecular weight.

### 3.2.4 Preparative IEF of DNT from DEAE-Sephacel chromatography

Fractions containing DNT from DEAE-sephacel chromatography were pooled and dialysed against distilled water to remove salts. Analysis by SDS-PAGE showed no difference in the protein profile or protein concentration, before or after dialysis. The pooled dialysed sample was prepared by addition of 2% (w/v) ampholytes in 50 ml distilled water, and loaded onto the Rotofor cell. Focusing the Rotofor cell required about 4 hours. The pH values of the Rotofor fractions are shown in Figure 3.5. The initial voltage and current were 386V, 25mA, with the power limited to 12W, while at equilibrium, the values were 658V, 15mA, and the power was limiting. Figure 3.4 shows that fractions 4 to 13, from a total of 19 fractions, contained a protein of 145kDa, which was equivalent to the size of DNT. DNT focused in fractions which contained a number of lower molecular weight proteins.

### 3.2.5 Preparative IEF of DNT from hydroxylapatite chromatography

Fractions containing DNT from hydroxylapatite chromatography were pooled and dialysed against distilled water. As was the case for DEAE-Sephacel chromatography fractions there was no difference in the profile or concentration of proteins in the sample, before or after dialysis (Figure 3.6). In addition, there was no
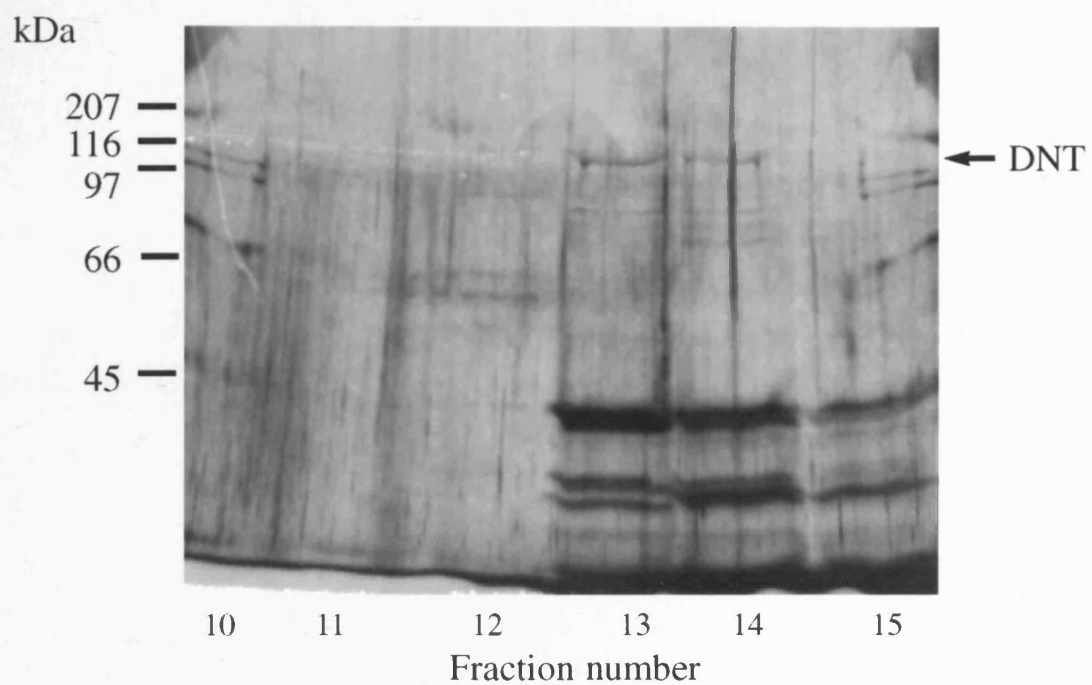
**Figure 3.3** Silver stained 8% SDS-PAGE of Superose 12 fractions 10-15, collected during the gel filtration of partially pure DNT in the presence of 1 M urea and 0.3 M $Na_2SO_4$. All lanes contain the same volume of sample.
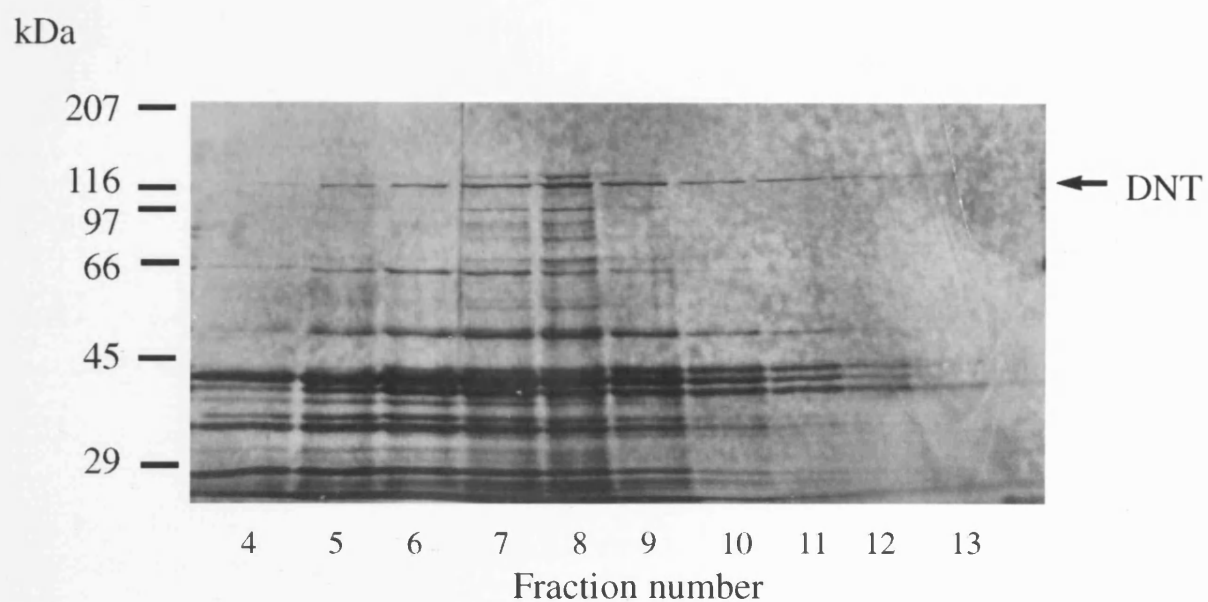
**Figure 3.4** Silver stained 8% SDS-PAGE of Rotofor fractions 4-13, in which partially pure DNT from DEAE-Sephacel chromatography was focused in the presence of 2% ampholytes, 3-10. All lanes contain the same volume of sample.
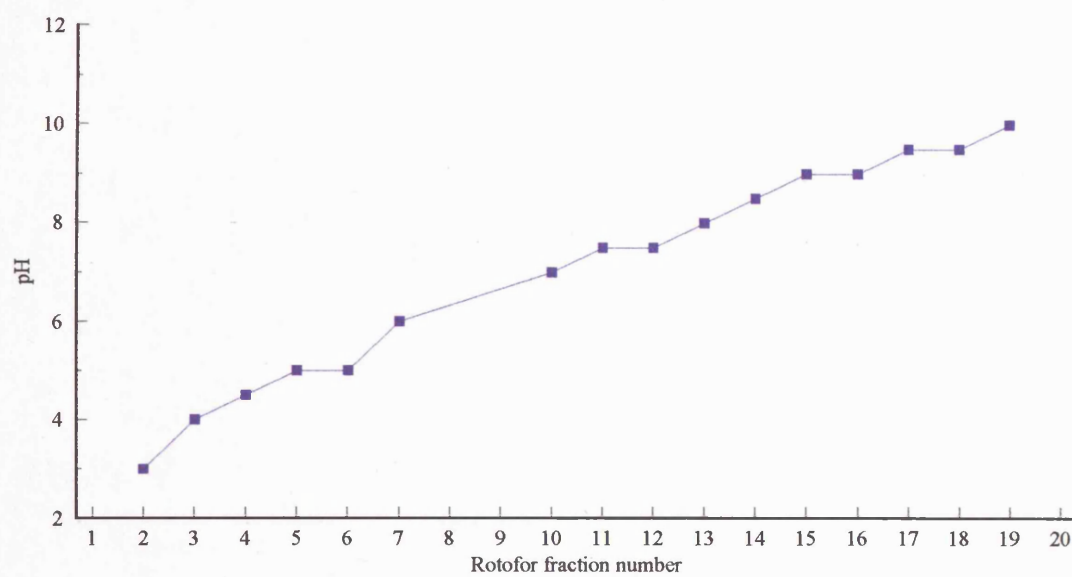
**Figure 3.5** The pH gradient of fractions taken from the Rotofor, in which partially purified DNT from DEAE-Sephacel chromatography was fractionated in the presence of 2% Biolytes, 3-10.
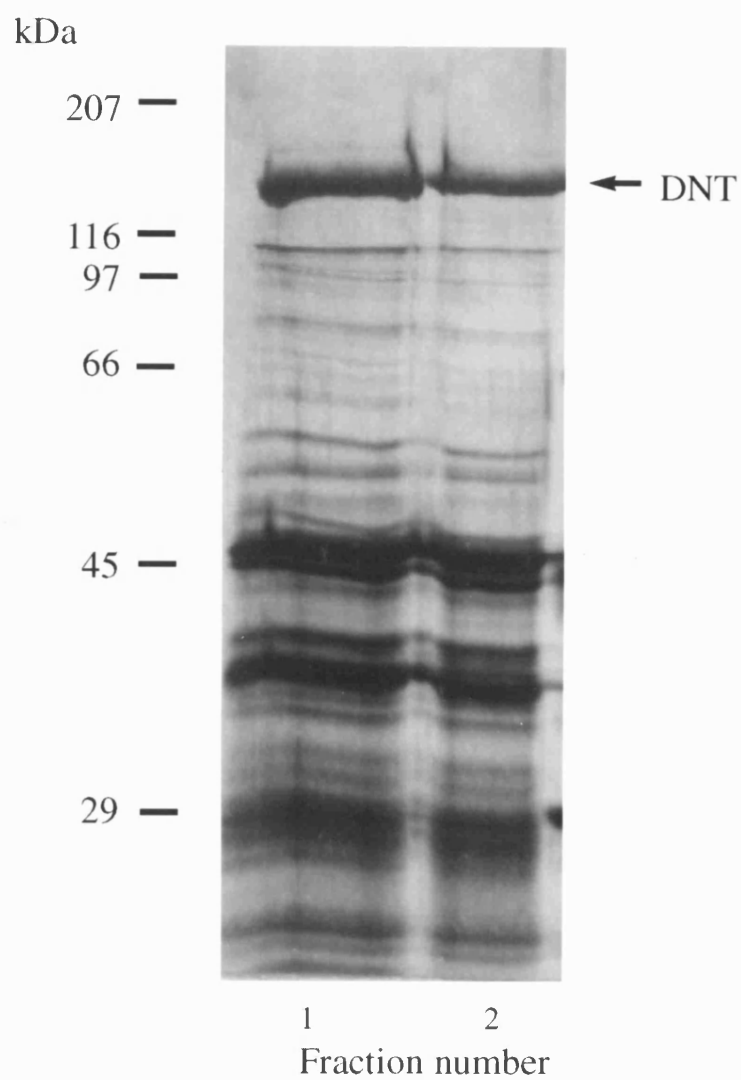
**Figure 3.6** Silver stained 8% SDS-PAGE of partially pure DNT from hydroxlapatite chromatography of *B. bronchiseptica* before (lane 1) and after (lane 2) dialysis against distilled water. All lanes contain the same volume of sample.

difference in cytotoxic activity for EBL cells before or after dialysis.

The sample was prepared by addition of 2% (w/v) ampholytes in 50 ml distilled water and loaded into the Rotofor cell. The average initial conditions were 441V, 22mA, and the power was limited to 12W. It took 4½ hours to focus the Rotofor cell and at equilibrium voltage and current were 713V, 14mA, and power limited. Analysis by SDS-PAGE showed there was no protein band at 145 kDa in any of the Rotofor fractions. Other lower molecular weight proteins in the original sample from hydroxylapatite chromatography were not seen by SDS-PAGE, despite the fact that a pH gradient formed as normal (Data not shown).

When fractions containing DNT from hydroxylapatite chromatography were focused in the presence of 3 M urea as well as the 2% ampholytes, a 145 kDa protein band was seen in Rotofor fractions 10-13 (Figure 3.7). These fractions, from pH 5.8 to 6.5, were cytotoxic on EBL cells (Figure 3.8), but after 4 days storage at 4°C, these fractions lost their cytotoxic activity, and the 145 kDa protein band could not be seen by SDS-PAGE. Dialysis of the DNT-containing fractions against phosphate buffer to remove the 3 M urea and 2% ampholytes also resulted in the loss of DNT from the samples.

To obtain better separation of DNT, the Rotofor fractions containing DNT were refractionated. Additional ampholytes were not added to the refractionated sample, so that the pH range corresponds to the pH range of the ampholytes contained in the pooled samples, creating a narrow pH gradient and allowing better separation. Refractionation of the DNT Rotofor samples resulted in the loss of DNT. None of the Rotofor fractions was cytotoxic to EBL cells, nor contained a 145 kDa protein when visualised by SDS-PAGE.

### 3.2.6 Stability of DNT purified by preparative IEF

I investigated why the Rotofor samples lost cytotoxic activity and the 145 kDa protein after 4 days storage at 4°C. The glass vials that had contained the Rotofor DNT samples were washed with SDS-PAGE loading buffer and heated for two minutes at 100°C. When analysed by SDS-PAGE the loading buffer contained a 145 kDa protein band (Figure 3.9).

The most stable condition for storage of the DNT Rotofor fractions was

investigated. Different types of containers were investigated: glass tubes, silicone-treated glass tubes and plastic tubes. None was more efficient than the others for storing DNT purified by the Rotofor (Figure 3.10). In glass vials there was a reduction in the loss of DNT activity when DNT was stored in the presence of glycerol (50% v/v final concentration). In all the containers DNT was more stable at -20°C in the presence of 50% glycerol, than at +4°C (Figure 3.10).
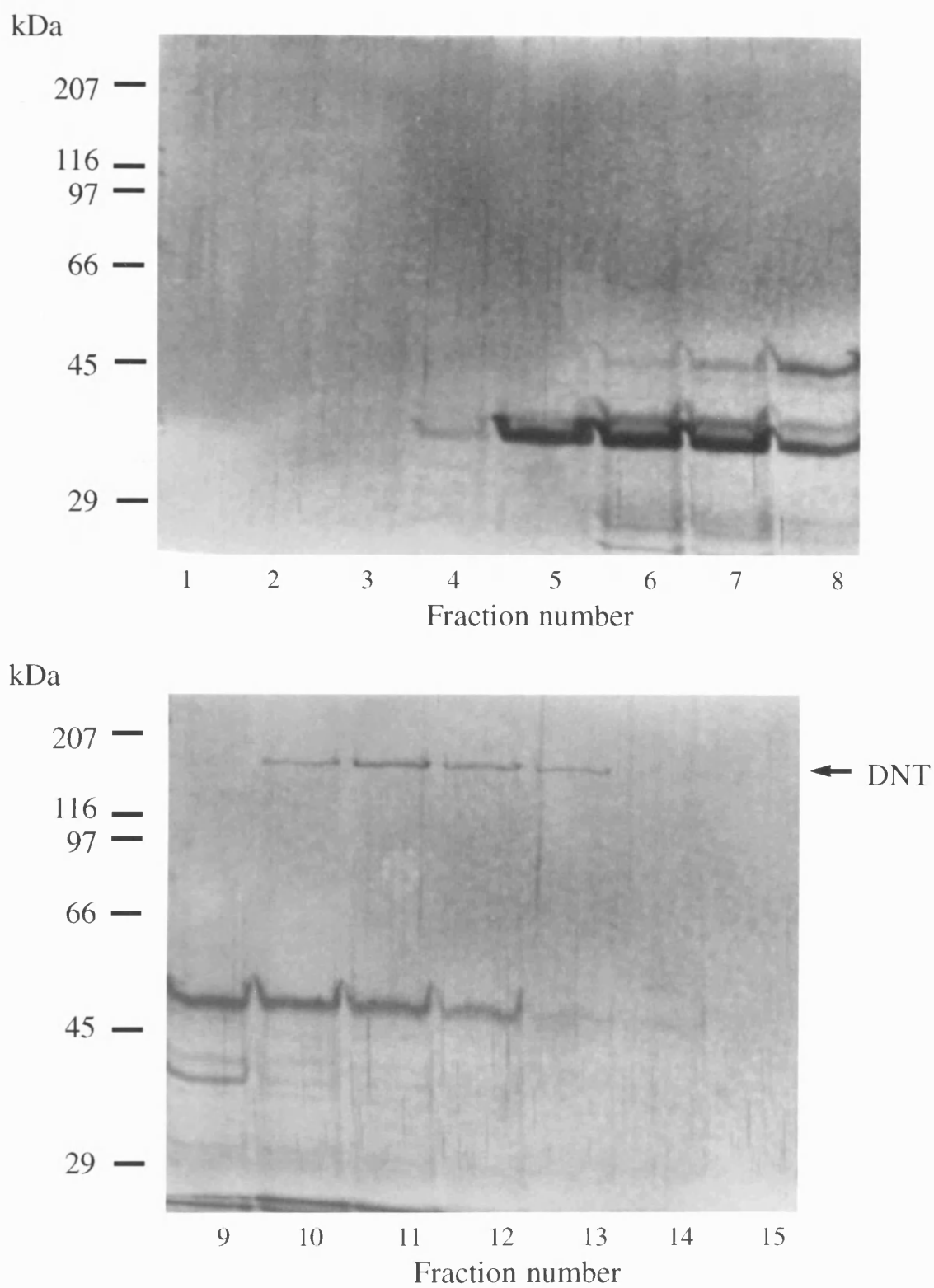
**Figure 3.7** Silver stained 8% SDS-PAGE of Rotofor fractions 1-15, in which partially pure DNT from hydroxylapatite chromatography was focused in the presence of 3 M urea and 2% ampholytes, 3-10. All lanes contained the same volume of sample.
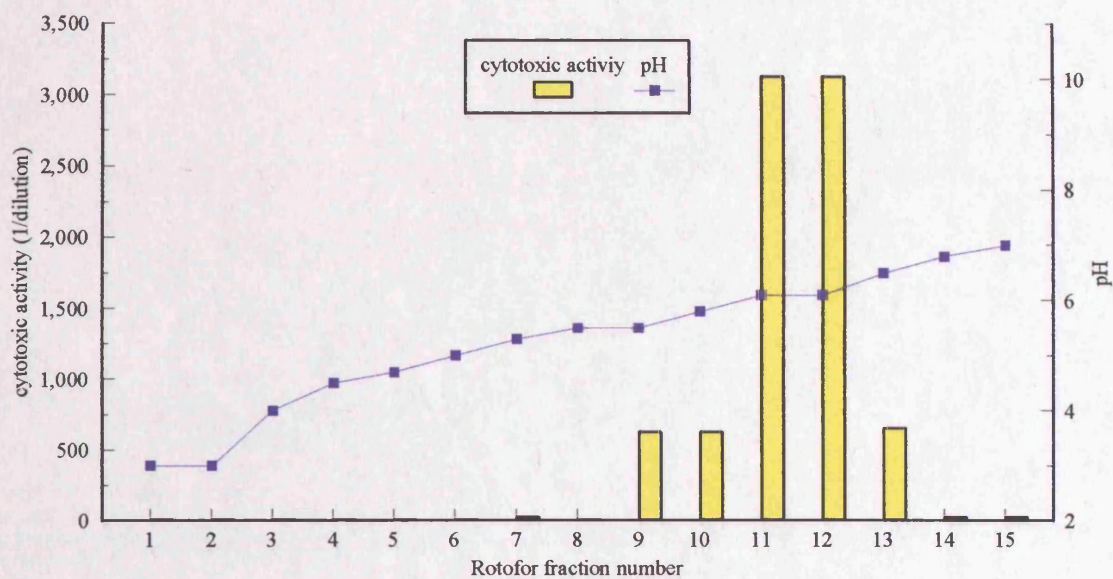
**Figure 3.8** The pH gradient and cytotoxic activity on EBL cells of 15 fractions isolated from the Rotofor, in which partially purified DNT from hydroxylapatite chromatography was fractioned in the presence of 2% Biolytes, 3-10, and 3 M urea. All samples were tested for cytotoxic activity in duplicate and diluted to an end point.
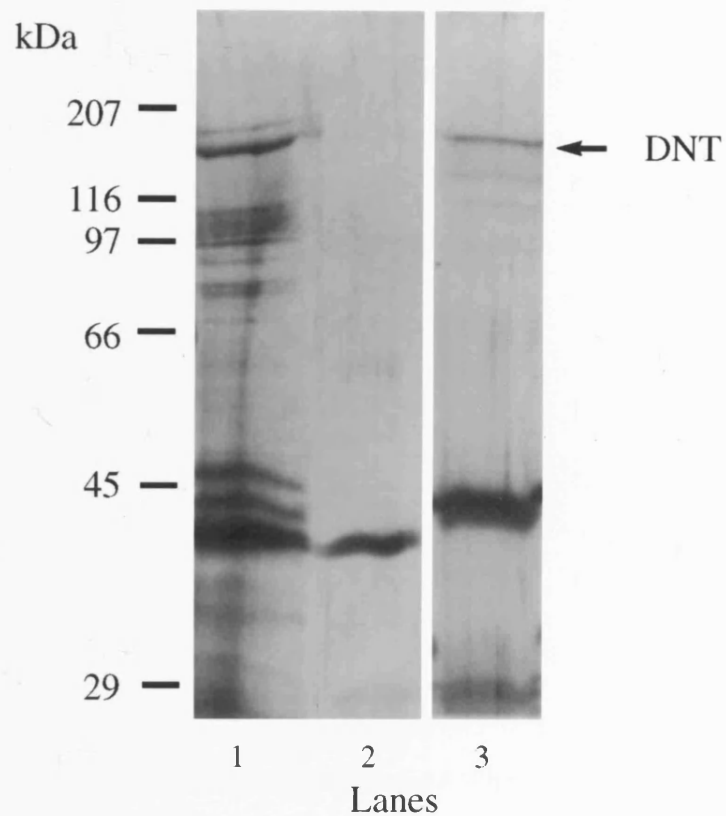
**Figure 3.9** Silver stained 8% SDS-PAGE of DNT samples stored in glass tubes at 4 °C. Lane 1, partially pure DNT from hydroxylapatite chromatography; lane 2, DNT-containing fraction from Rotofor after 4 days storage; Lane 3, SDS-PAGE loading buffer, boiled in empty glass tube used to store DNT purified from Rotofor in lane 2. All lanes contained the same volume of sample.
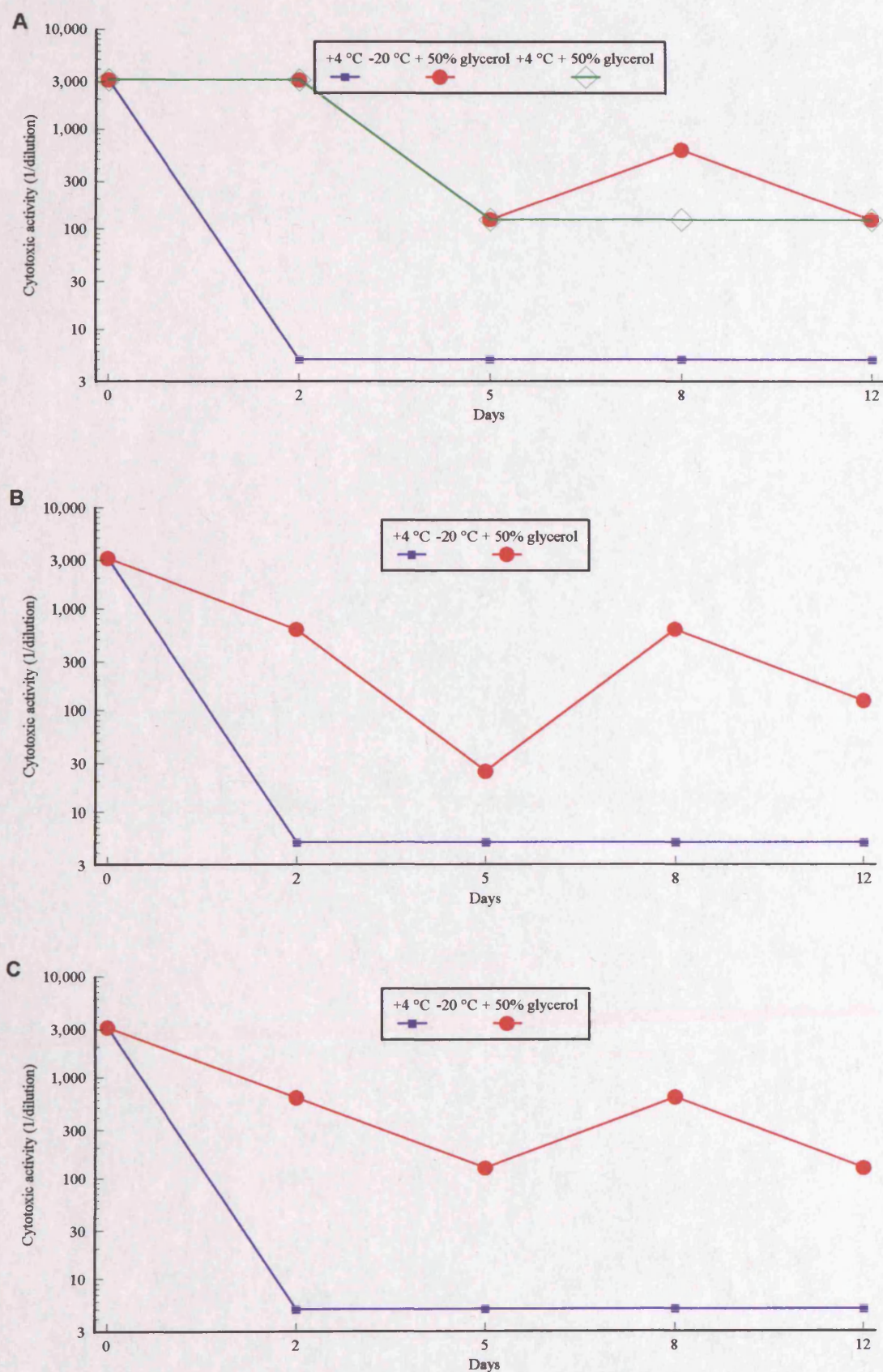
**Figure 3.10** Stability of DNT, purified using a Rotofor, stored at 4°C or -20°C with 50% (v/v) glycerol in various containers: A, glass vials; B, silicone-treated glass vials; C, plastic tubes. All samples were tested for cytotoxic activity on EBL cells in duplicate and diluted to an end point.

## 3.3 Discussion

In this work several methods have been investigated in an attempt to improve the purification scheme for DNT (see Figure 3.11).

The removal of nucleic acids and acidic proteins by Kurimover C-7 did not improve the purification of DNT because the majority of the nucleic acids were not removed, and DNT was lost during the precipitation of the proteins.

The effect of urea was investigated in several methods to see if it would prevent aggregation of DNT and increase the stability of the toxin. The use of 1 M urea made no difference to the dialysis of partially purified DNT, as DNT did not seem to stick to dialysis tubing without urea, contrary to the results of Garrod (1994). Dialysis was successfully used in the preparation of partially purified DNT for the Rotofor cell.

Research by Garrod (1994) showed that gel filtration failed to separate DNT from other cellular proteins. DNT was eluted from the column later than expected with the other lower molecular weight proteins. This result may have been due to aggregation of DNT to the other cellular components, or to the column matrix causing it to be eluted later. However, the addition of 1 M urea and 0.3 M $Na_2SO_4$ to the column buffer in gel filtration did not prevent this aggregation, and DNT was eluted with other lower molecular weight proteins. This method was pursued no further as it had been investigated previously, and the other cellular proteins in the fractions were much more concentrated than DNT.

A new method to purify DNT on the basis of its pI using the Rotofor cell was investigated. The initial experiments with the Rotofor were carried out with DNT from DEAE-Sephacel chromatography, since the purification scheme would have been shorter if DNT could have been separated at this stage. But DNT from DEAE-Sephacel chromatography focused in the Rotofor over a wide pH range, 5.5-8.0, and many other proteins also focused in this pH range. Therefore, it was not possible at this stage of the purification scheme to separate DNT from other cellular proteins using preparative IEF.

When cleaner fractions of DNT from hydroxylapatite chromatography were focused in the Rotofor, DNT was not seen by SDS-PAGE analysis. It was assumed that the loss of proteins was due to precipitation at their pI as reported by Egen *et al.*
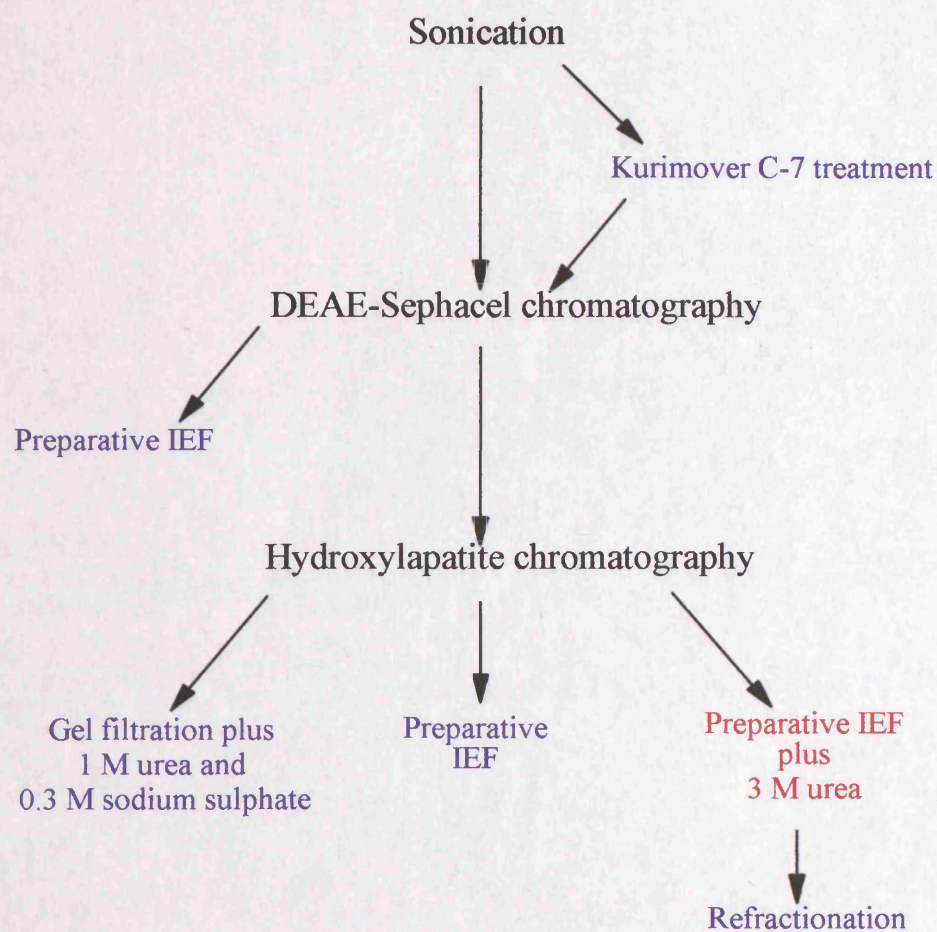
**Figure 3.11** Modifications to the procedure of Garrod (1994) for the purification of DNT from *B. bronchiseptica*. Successful modification in red, unsuccessful modifications in blue.

(1988). The addition of 3 M urea (Egen *et al.*, 1988; Garfin, 1990) to the sample during IEF prevented the precipitation. DNT focused in a pH range 5.8-6.5 similar to its reported pI (Endoh *et al.*, 1986c; Kume *et al.*, 1986; Horiguchi *et al.* 1989). These fractions contained very small amounts of lower molecular weight proteins and a protein of 45 kDa. The fractions containing DNT were cytotoxic on EBL cells, but were about 25-fold less active than the loaded sample. The addition of 3 M urea to partially pure DNT from hydroxylapatite chromatography did not reduce the cytotoxicity of DNT. The data shown for the purification of DNT using the Rotofor is representative of approximately ten separate purification procedures performed during this work.

Attempts to purify DNT further by refractionation of the Rotofor DNT fractions were unsuccessful due to the loss of DNT. This was possibly due to the precipitation of DNT or aggregation of DNT to the Rotofor apparatus, or due to the dilution of the sample or a combination of all these possibilities. At this low level of DNT expression observed it will be difficult to purify DNT any further due to its low concentration, instability and aggregation.

It was necessary to remove the 3 M urea and 2% ampholytes from the DNT samples because they might have interfered with further analysis of the samples, e.g. in protein or cell assays. Dialysis was the most common and simplest method used to remove ampholytes from samples. Dialysis of the Rotofor DNT fractions against PBS resulted in the loss of DNT, possibly due to aggregation of the toxin to the dialysis tubing. Other methods such as chromatography techniques, e.g. gel filtration and ion-exchange, used to separate the ampholytes from the sample were not investigated because of the high probability of loss of DNT due to aggregation of the toxin. Unfortunately, this meant that the samples could not be assayed for protein content so the specific activities and yields for this purification procedure could not be calculated.

Storage of the Rotofor DNT fractions at 4°C showed that the toxin was very unstable. The cytotoxic activity and the 145 kDa protein were lost after 4 days storage. A 145 kDa protein was visualised when the empty glass tubes that had contained the DNT fractions were washed and boiled with SDS-PAGE loading buffer and analysed by SDS-PAGE. This suggested that loss of DNT was due to adsorption of the toxin to the glass vials during storage. Adsorption of proteins to glass or plastic

during purification or study of proteins at low concentrations is a serious problem (Suelter and Deluca, 1983; Pande and Murthy, 1994). A variety of techniques have be used to reduce the loss of proteins from dilute solutions by adsorption. Some researchers modify the solvent whilst others modify the container or use an alternative container. The addition of bovine serum albumin (BSA) to a solvent during the purification of a protein is a common method used to prevent protein loss, but seems to defeat the purpose of purification. The different containers used to store the Rotofor DNT fractions did not affect the adsorption of the toxin. However, the addition of glycerol (50% v/v final concentration) did reduce the loss of DNT by adsorption. Suelter and Deluca (1983) reported that adding glycerol or Triton X-100 was effective in preventing the loss of proteins by adsorption. They suggested that glycerol is excluded from the immediate domain of the protein molecule causing an enhanced ordering of the solvent which favours a more folded, compacted state of the protein molecule. This folding reduced the nonpolar surface area on the molecule which in turns reduced interaction of the protein with the glass or plastic surface.

A relatively pure sample of active DNT was prepared using the Rotofor cell to separate DNT from other cellular proteins on the basis of its pI. The addition of 3 M urea was necessary to prevent the loss of DNT either by precipitation or adsorption. This method gave reliable and reproducible results, but unfortunately the purified DNT was in an unsuitable buffer and unstable. The 3 M urea and ampholytes could not be removed from the purified DNT, and so although the DNT had been separated from the cellular proteins, it was contaminated with other substances which interfered with further analysis of the toxin. Also, adsorption of DNT to the storage containers, even in the presence of 50% glycerol, meant that the DNT samples could not be stored and had to be used immediately. Due to these problems, DNT was not regularly purified using the Rotofor.

Many researchers have been unsuccessful in purifying DNT from *Bordetella* species using a wide variety of techniques. The instability of the toxin and its adsorption to other cellular components and apparatus has hindered its purification. However, the production of monoclonal antibodies against DNT (see Chapter 6) will allow us to investigate affinity purification as a possible new technique to purify DNT.

*Chapter 4*

*Partial purification of recombinant*

*dermonecrotic toxin*

## 4.1 Introduction

Determining the molecular mode of action of DNT and its role in disease have been hindered, compared to other virulence factors of *Bordetella* species, due to difficulties in the purification of the toxin. Identification and cloning of the gene encoding DNT would be a great advantage in further characterisation of the toxin. Cloning and expression of DNT in a well-characterised *E. coli* system would allow definite assignment of particular biological activities to the toxin. Also, expression in a recombinant system could yield larger quantities of DNT compared to the wild type.

DNT was one of the first virulence factors to be identified for *B. pertussis* by Bordet and Gengou in 1909, but until very recently it had not been cloned. Transposon mutagensis with Tn5*lac* produced a DNT-negative *B. pertussis* strain, BPM1809 (Weiss *et al.*, 1989). This strain was characterised and found to be deficient only in the production of DNT. Strain BPM1809 did not produce characteristic dermonecrotic lesions when injected subcutaneously in mice. However, the DNT-negative strain was unimpaired in its ability to cause lethal infection in infant mice (Weiss and Goodwin, 1989). The DNA region around the Tn5*lac* insertion was cloned in three overlapping fragments, and the nucleotide sequence determined (Walker and Weiss, 1994). The open reading frame was thought to encode the DNT gene. However, since the gene was not cloned on a single insert and the product was not expressed, it was not proven definitively that this was the structural gene for DNT. Walker and Weiss (1994) determined by Southern analysis that the restriction maps of the putative DNT gene were nearly identical in *B. pertussis*, *B. bronchiseptica* and *B. parapertussis*, but the sequence was not present in toxigenic *B. avium* strains. There was homology between the amino acid sequence of DNT and the *E. coli* cytotoxic necrotizing toxins, CNF1 and CNF2. However, the DNT sequence showed no homology to PMT (see Section 1.4.2.6).

Recently, Pullinger *et al.* (1996) cloned and expressed the DNT gene of *B. bronchiseptica* for the first time. A cosmid library of random fragments of genomic DNA from *B. bronchiseptica* was made and screened with oligonucleotides from the *B. pertussis* DNT gene sequence. The complete DNT gene was subcloned into a high-copy-number plasmid, pBluescript, and this resulted in expression of rDNT. Sequence analysis of the DNT gene showed that it was 99% homologous to the DNT gene of

*B. pertussis*. This confirmed that the sequence described by Walker and Weiss (1994) represented the *B. pertussis* DNT gene. Crude lysates from *E.coli* XL1-blue/bluescript (pDNT1) (refered to as DNT1 in this work) were cytotoxic to EBL cells, dermonecrotic in the infant-mouse dermonecrosis assay, and stimulated DNA synthesis in Swiss 3T3 cells.

The DNT genes of *B. bronchiseptica* and *B. pertussis* encode for a purine nucleotide-binding motif, the P-loop (Saraste *et al.*, 1990). Comparison of the sequences of nucleotide-binding proteins showed that another linked motif that confers GTP specificity (Dever *et al.*, 1987) is absent from DNT. This suggested that DNT may be an ATP-binding protein. The possible role of this nucleotide-binding motif of DNT was investigated by making base substitutions in the conserved lysine residue, which is thought to interact directly with one of the phosphate groups of the nucleotide. Site-directed mutagenesis of the conserved lysine residue to alanine resulted in strain K1310A which was noncytotoxic on EBL cells (Pullinger *et al.* 1996) .

The aim of the work in this chapter was to obtain pure recombinant DNT (rDNT) from the *E. coli* clone, DNT1. This would allow characterisation of the toxin and elucidation of its role in disease, and ultimately its mode of action. The purification of DNT has proven difficult (see Chapter 3), mainly due to the characteristic instability of the toxin, and its poor and unreliable recovery. Garrod (1994) developed a quick, reliable, and reproducible method for the partial purification of DNT from *B. bronchiseptica*. This purification method was used as the basis for developing a purification method for rDNT. Like DNT, rDNT was cytotoxic to EBL cells and this assay was used to detect the presence of the toxin throughout the purification procedure. SDS-PAGE was used to analyse the protein content of samples and to detect the rDNT protein.

To comply with ACGM regulations all work with DNT1 had to be carried out at category 3+ conditions. Protein extracts from DNT1 could be moved to a category 2 laboratory, as long as the samples were cell-free, nucleic acid-free, and sterile.

## 4.2 Results

### 4.2.1 Bacterial strains and growth conditions

The recombinant strain DNT1 (*E. coli* XL1-blue/bluescript(pDNT1)) was used as the source of rDNT in all the biological experiments.

In previous experiments, Pullinger *et al.* (1996) had used 10 ml broth culture incubated overnight at 37°C, with shaking at 180 rpm. These were lysed the following day and tested on EBL cells. However, for the purposes of developing a useful purification method for rDNT, the growth of DNT1 was scaled up. Half (5 ml) of an overnight culture, prepared as above, was used to inoculate 300 ml LB broth, incubated at 37°C, and shaken at 180 rpm. In order to assay the amount of toxin produced at different stages during growth of strain DNT1, 10 ml samples were taken at time points to measure $OD_{550}$ and viable counts, followed by sonication of the samples to analyse proteins by SDS-PAGE and cytotoxic activity on EBL cells.

Five hours after inoculation the bacterial culture was at the end of its log phase of growth. At this time a protein band of approximately 145 kDa, the size of rDNT, was shown by 5% SDS-PAGE (Figure 4.1). The intensity of the 145 kDa protein band increased until 9 hours after inoculation, when the bacterial culture was in the death phase, as shown by the decrease in viable counts. SDS-PAGE showed that the intensity of the 145 kDa protein band remained constant for up to 24 hours after inoculation. The sonicated cell extract taken 5 hours after inoculation was cytotoxic to EBL cells, and the cytotoxic activity increased in the sonicated cell extracts taken up to 9 hours after inoculation (Figure 4.2). Twenty-four hours after inoculation the cytotoxic activity of the sonicated cell extract decreased 125-fold even though the rDNT band was still visible by SDS-PAGE.

IPTG induces expression of the *lacZ* gene in the bluescript plasmid. However, the addition of 1 mM IPTG to overnight cultures of DNT1 grown in LB broth did not effect the production of rDNT (data not shown).

The same experiment was repeated using 2 × YT medium. Five hours after inoculation a 145 kDa rDNT protein band first appeared and the sonicated cell extract became cytotoxic to EBL cells (Figures 4.3 and 4.4). The intensity of the rDNT band shown by SDS-PAGE increased until 9 hours after inoculation and remained constant for up to 24 hours. The cytotoxic activity of the sonicated cell extract also increased

94
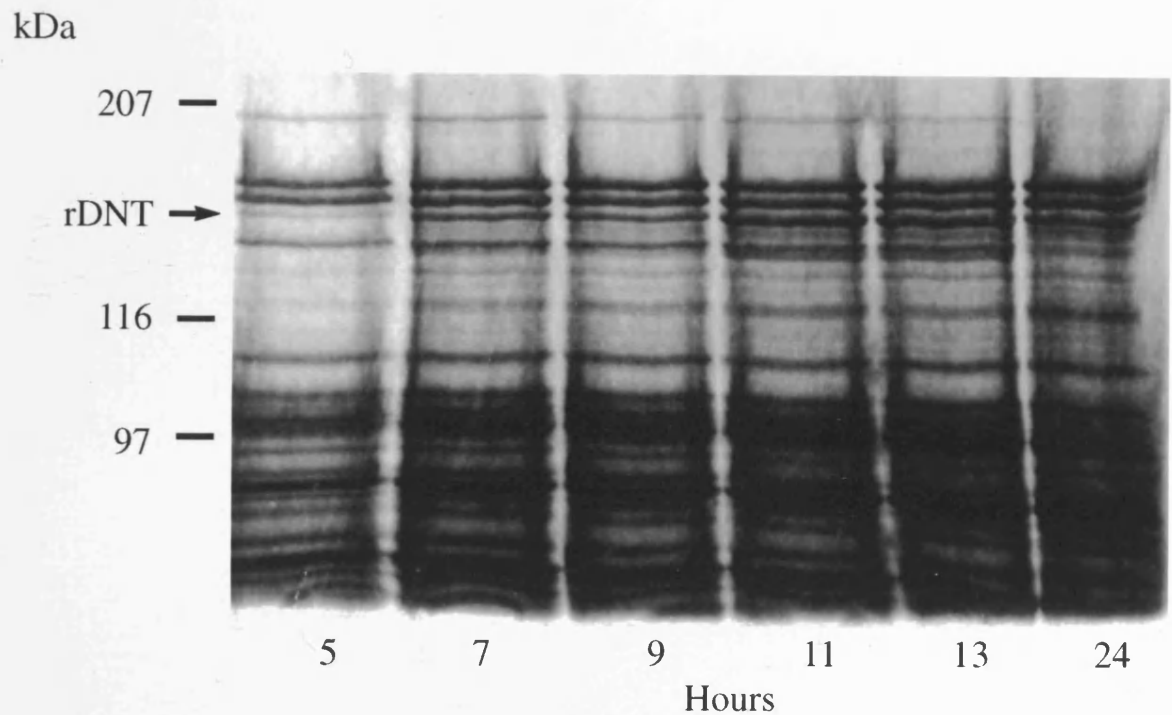
**Figure 4.1** Silver stained 5% SDS-PAGE of sonicated cell extracts of strain DNT1 grown in LB broth. Samples for sonication were taken at various time points after inoculation. All lanes contain the same volume of sample.
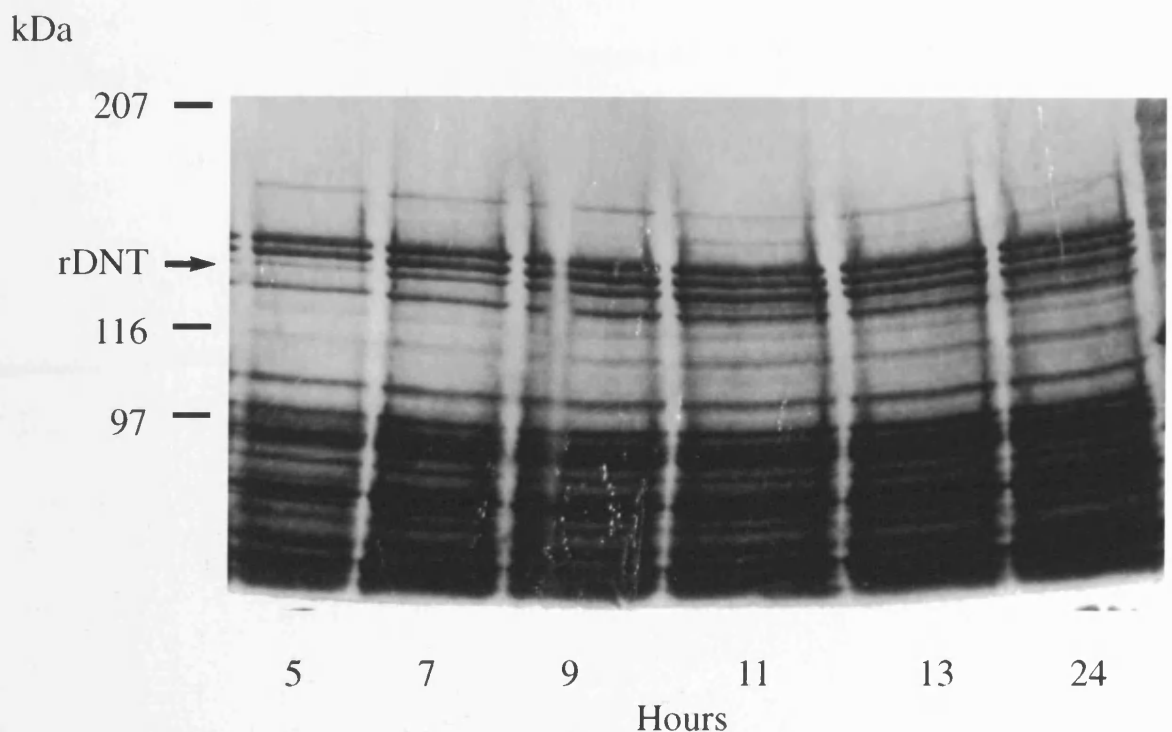


**Figure 4.3** Silver stained 5% SDS-PAGE of sonicated cell extracts of strain DNT1 grown in 2 x YT medium. Samples for sonication were taken at various time points after inoculation. All lanes contain the same volume of sample.
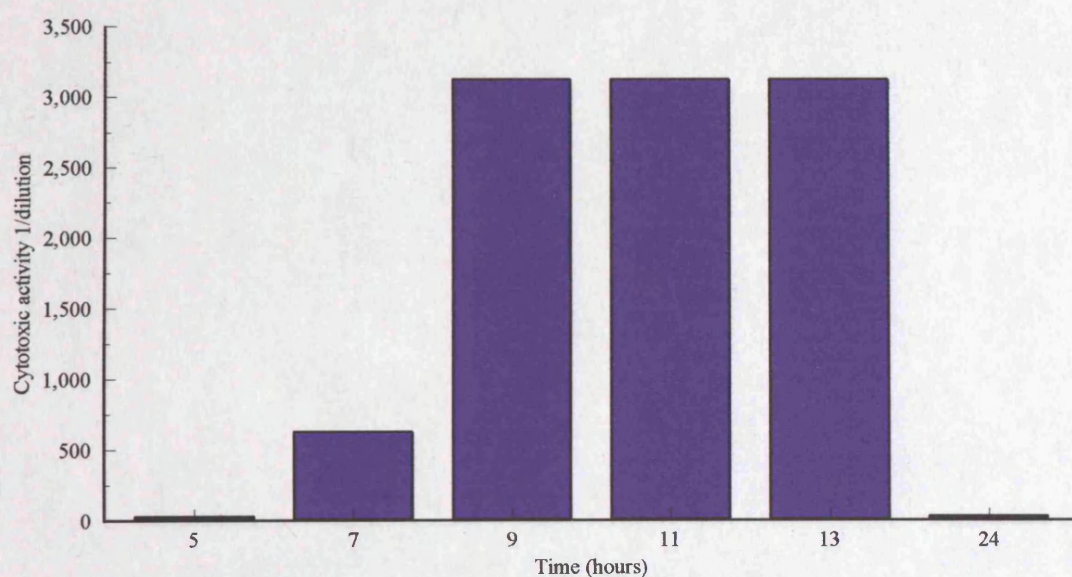
**Figure 4.2** The cytotoxic activity on EBL cells of sonicated cell extracts taken at various time points from a culture of strain DNT1 grown in LB broth. All samples were tested in duplicate and diluted to an end point.
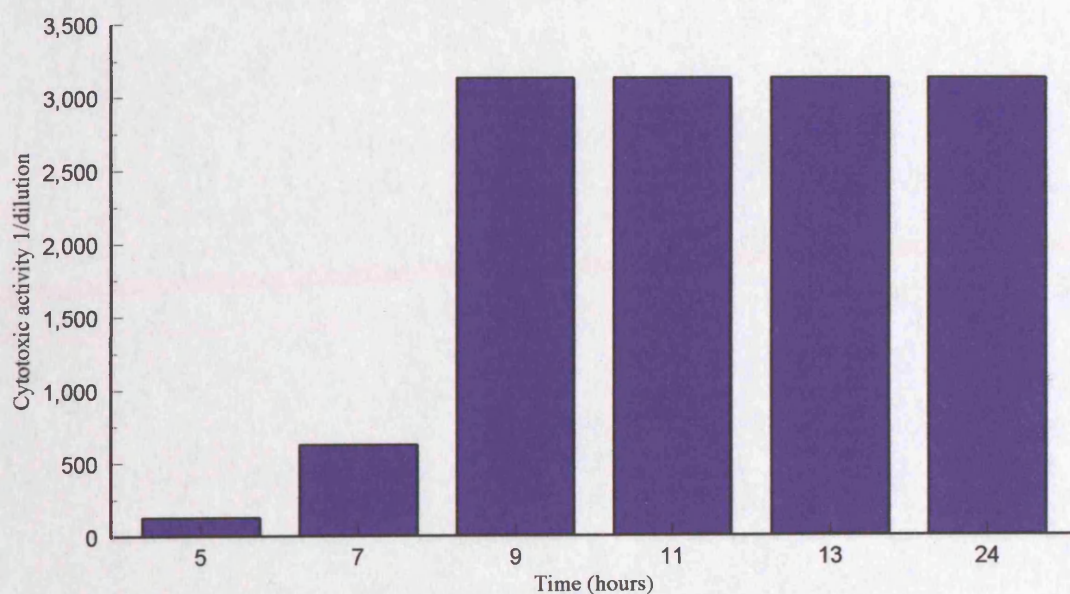


**Figure 4.4** The cytotoxic activity on EBL cells of sonicated cell extracts taken at various time points from a culture of strain DNT1grown in 2 x YT medium. All samples were tested in duplicate and diluted to an end point.

up to 9 hours, but unlike cells grown in LB broth, the activity remained constant for 24 hours.

### 4.2.2 Preparation of cell free extract

#### 4.2.2.1 Preparation of cell free extracts by enzymatic lysis

Culture supernatant from DNT1 was not cytotoxic to EBL cells (data not shown). Therefore, rDNT is an intracellular protein, like the wild type DNT (Cowell *et al.*, 1979), and had to be released from the cells of *E. coli*. Sonication was used to release DNT from *B. bronchiseptica*. But due to safety concerns, because this work was carried out at category 3+ conditions, it was decided to lyse cells of *E. coli* enzymatically.

DNT1 was grown as described above in 300 ml of 2 × YT medium for 12 hours. The bacterial cells were harvested and resuspended in lysozyme lysis buffer (5 mM EDTA, 50 mM Tris-Cl (pH 7.2), 100 mM NaCl, 0.1% (v/v) toluene and 50 µg ml$^{-1}$ lysozyme), and left at room temperature for 4 hours. After the cell debris was removed, the resulting cell lysate was cytotoxic to EBL cells to 1/15625 end point dilution, and 5% SDS-PAGE showed a 145 kDa protein band (Figure 4.5).

The crude lysate could not be used directly on the DEAE-Sephacel chromatography column because the lysis buffer contained EDTA, toluene, and NaCl, which would interfere with the binding of proteins to the column matrix. Therefore, the DNT1 crude lysate was dialysed overnight at room temperature against 50 mM Tris/HCl pH 7.5, the ion exchange column buffer used in the purification of *B. bronchiseptica* DNT. After dialysis, the DNT1 crude lysate had a light precipitate, and was cytotoxic on EBL cells only to a 1/25 dilution, and no rDNT band could be seen by SDS-PAGE (Figure 4.5). When the DNT1 crude lysate was dialysed against 50 mM Tris pH 7.5 containing 50 mM NaCl, rDNT was not lost and there was no precipitation. However, when this dialysed lysate was loaded onto the DEAE-Sephacel column, rDNT did not bind and was washed off the column (data not shown).

To try to resolve these problems, DNT1 was lysed with buffer containing either no NaCl or 10 mM NaCl. Both buffers lysed *E. coli* cells, but the rDNT band was only faintly visible by 5% SDS-PAGE (Figure 4.5) and the crude lysates were cytotoxic on EBL cells only to a 1/25 dilution. After dialysis against 50 mM Tris pH

7.5, no rDNT band was visible by SDS-PAGE and the samples were not cytotoxic to EBL cells.

### 4.2.2.2 Preparation of cell extracts by sonication

Enzymatic lysis was not successful in the preparation of cell extracts of DNT1, and so sonication was used. *E. coli* cells are easier to disrupt than those of *Bordetella* species so sonication could be kept to a minimum, and appropriate safety precautions taken. Sonication was performed in a Class II biological safety cabinet within a sealed container. The horn of the sonicator was immersed deep enough below the surface of the suspension to ensure that the mixture did not foam or form aerosols. The possibility of aerosol formation could be further reduced by decreasing the power and increasing the time of sonication. After sonication all equipment and glassware used during the procedure were fumigated in the safety cabinet before being removed.

DNT is known to be heat labile at 56°C (Munzo, 1971), and therefore the temperature had to be carefully controlled to avoid denaturation of the protein. Suspensions were cooled to 4°C before sonication and kept on salted ice throughout. The temperature was also minimised by allowing cooling between pulses of sonication. The low temperature also reduced the risk of proteolysis by proteases released from bacterial cells during sonication.

Strain DNT1 was resuspended in 100 ml of 50 mM Tris pH 7.5, and cooled to 4°C. The suspension was sonicated at 70 W for 10 minutes in 2 minute pulses with 2 minute intervals for cooling. Samples were taken after every pulse and the sonicated cell extracts analysed by SDS-PAGE and on EBL cells. SDS-PAGE showed that after the first 2 minutes of sonication the cells had been broken, releasing a 145 kDa protein (Figure 4.6). After 8 minutes of sonication the 145 kDa band was not visible by SDS-PAGE. The sonicated cell extract was cytotoxic to EBL cells to a 1/15625 end point dilution after 2 minutes sonication, but after 8 minutes sonication the cytotoxic activity decreased 5 fold to a 1/3125 end point dilution.

The results from these experiments were used to set up a standard procedure for the production of DNT1 cell-free sonicated cell extract, a 100 ml volume of DNT1 cell suspension was cooled to 4°C and sonicated at 70 W for 2 minutes. This was a reliable method for obtaining a cell-free sonicated cell extract which was used in
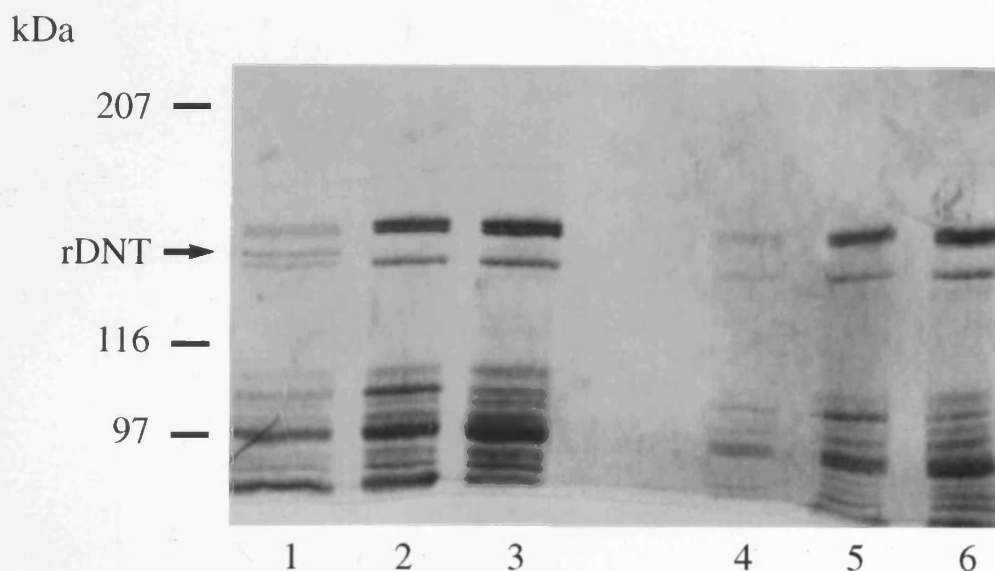
**Figure 4.5** Silver stained 5% SDS-PAGE of cell lysates from DNT1. Lanes 1 & 4, cell lysate prepared with 100 mM NaCl lysis buffer; lanes 2 & 5, cell lysate prepared with 10 mM NaCl lysis buffer; lanes 3 & 6, cell lysate prepared with 0 mM NaCl lysis buffer. The samples in lanes 1, 2 & 3 are the cell lysates before dialysis and lanes 4, 5 & 6 are the cell lysates after dialysis against 50 mM Tris pH 7.5.
All lanes contain the same volume of sample.
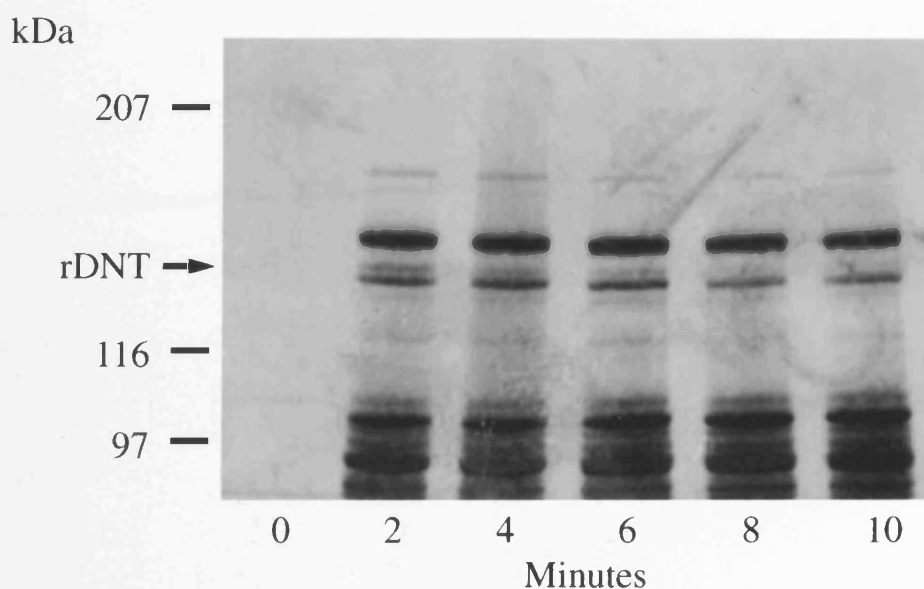


**Figure 4.6** Silver stained 5% SDS-PAGE of DNT1 sonicated cell extracts. At various time points during the sonication of 100 ml suspension of strain DNT1 samples were taken for analysis.
All lanes contain the same volume of sample.

subsequent purification procedures.

### 4.2.3 RNAase and DNAase treatment of sonicated cell extract

To comply with ACGM regulations the DNT1 sonicated cell extract had to be free of nucleic acids before it was removed from the category 3+ laboratory. The sonicated cell extract was centrifuged to remove cell debris and then treated with DNAase and RNAase, each at 10 µg ml$^{-1}$, for 2 hours at 37°C. Despite the potential for loss of rDNT at 37°C due to proteolysis or heat labile characteristics, DNAase and RNAase treatment did not reduce the cytotoxic activity of the sonicated cell extracts (data not shown).

Following DNAase and RNAase treatment the sonicated cell extract was centrifuged and filtered through a 0.22 µm pore-size membrane. This was necessary to sterilise the extract so it could be taken from the category 3+ laboratory, and to remove any cell debris which could block the chromatography column in subsequent purification steps.

### 4.2.4 Ion-exchange chromatography of sonicated cell extract from DNT1 on a DEAE-Sephacel column

The sonicated cell extract of DNT1 was loaded onto and eluted from the DEAE-Sephacel column under exactly the same conditions as the sonicated cell extract of *B. bronchiseptica*. However, the linear ionic gradient that was successful in separating wild-type DNT, resulted in rDNT being eluted from the DEAE-Sephacel column in all fractions collected (data not shown).

A stepwise ionic strength gradient produced a higher resolution of protein separation. A typical elution profile of proteins separated on the DEAE-Sephacel column using a stepwise ionic strength gradient is shown in Figure 4.7. The rDNT eluted from the DEAE-Sephacel column at approximately 150-180 mM NaCl. The fractions containing rDNT were identified as those with cytotoxic activity on EBL cells to a 1/15,625 dilution (Figure 4.8). The cytotoxic activity correlated with the appearance of a protein band at 145 kDa visible by SDS-PAGE.

In a representative run for which the data are shown, fractions 13-16 were pooled (approx. 20 ml) for further purification using hydroxylapatite chromatography.

100
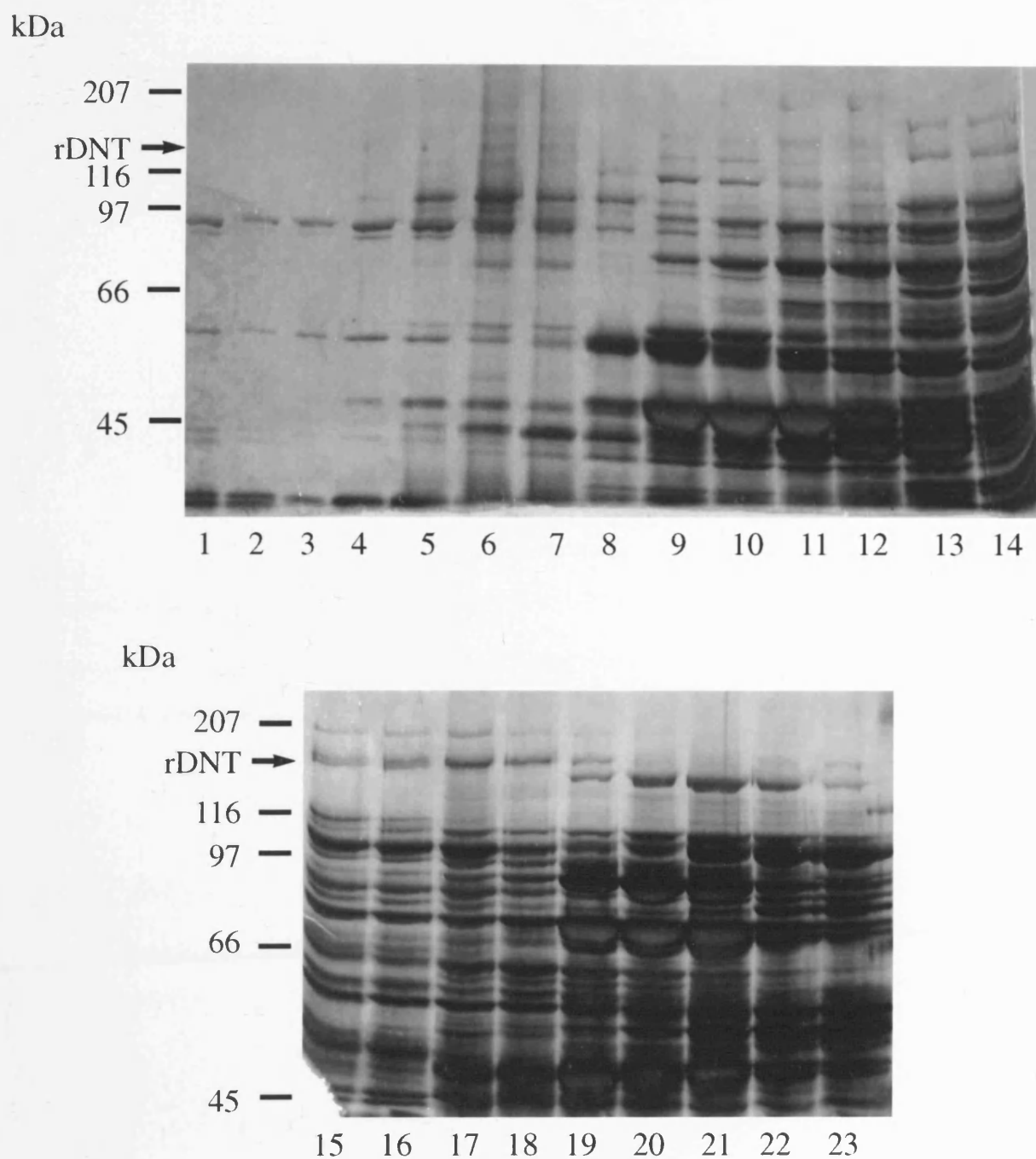
**Figure 4.7** Silver stained 8% SDS-PAGE of DEAE-Sephacel column fractions collected during purification of rDNT from strain DNT1. All lanes contain the same volume of sample.
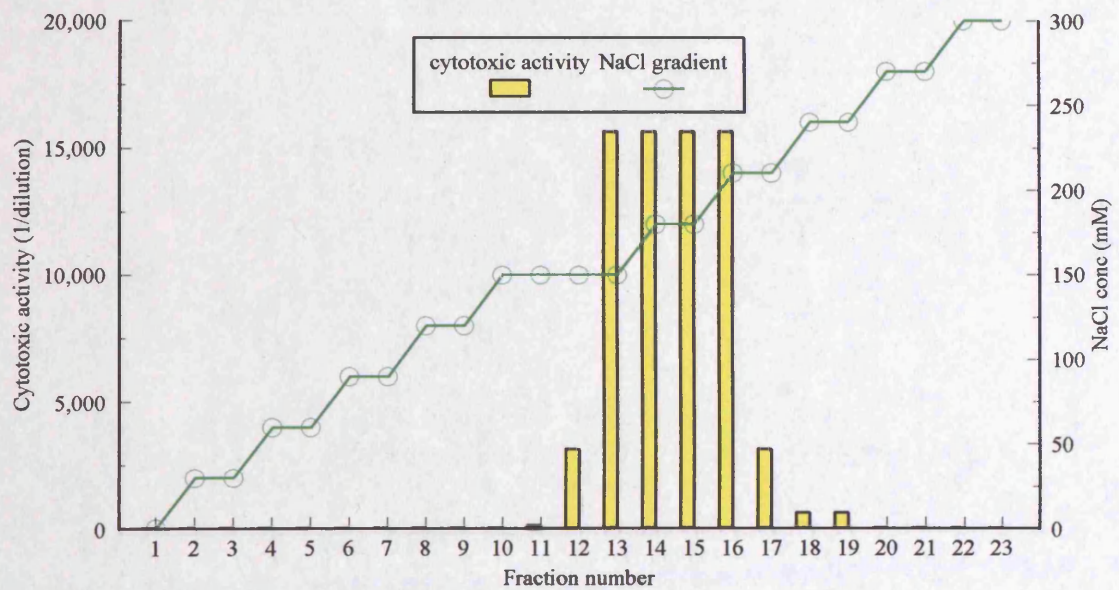
**Figure 4.8** The cytotoxic activity on EBL cells of DEAE-Sephacel column fractions collected during the purification of rDNT from strain DNT1, eluted with a step-wise NaCl gradient. All samples were tested in duplicate and diluted to an end point.

## 4.2.5 Affinity chromatography of DNT1 samples containing rDNT on a hydroxylapatite column

A typical elution profile of proteins separated on the hydroxylapatite column is shown in Figure 4.9. The rDNT eluted from the hydroxylapatite column at approximately 180 mM $K_2PO_4$. The fractions containing rDNT were cytotoxic to EBL cells to 1/78,125 dilution (Figure 4.10), and showed a protein band of 145 kDa by SDS-PAGE (Figure 4.9). In the representative run fractions 9 to 17 contain rDNT, and most rDNT was eluted from the column in fractions 13-17.

Recombinant DNT fractions from the hydroxylapatite column were purer than those from the DEAE-sephacel column, although other proteins still co-purified with rDNT. Fractions 14-17 from the hydroxylapatite column which contained high levels of rDNT were considered partially pure preparations of rDNT.

## 4.2.6 Degree of purification of partially pure preparations of rDNT

Figure 4.11 shows SDS-PAGE analysis of samples from each stage of purification, and Table 4.1 shows the specific activity of rDNT from each stage of this representative purification. The total protein in samples at each stage of the purification was determined using the Pierce BCA protein assay. Partially pure rDNT contained 2% of the total protein present in the sonicated cell extract. The activity at each stage of the purification was measured by diluting the samples to an end point on EBL cells. From the total protein estimations and the level of activity, the specific activity at each stage of purification was calculated. The specific activity for this representative partial purification of rDNT was 62 times that of the sonicated cell extract.

The relative concentration of rDNT in the partially purified fractions from the hydroxylapatite column was difficult to determine due to the co-purifying proteins. Two approaches were taken: i) from silver stained gels, the rDNT band was 10% of the total protein in the hydroxylapatite fraction, giving an estimate rDNT concentration of 33 $\mu g$ $ml^{-1}$; ii) from silver stained gels, the intensity of the rDNT band was compared with protein marker bands of known concentration, giving an estimate rDNT
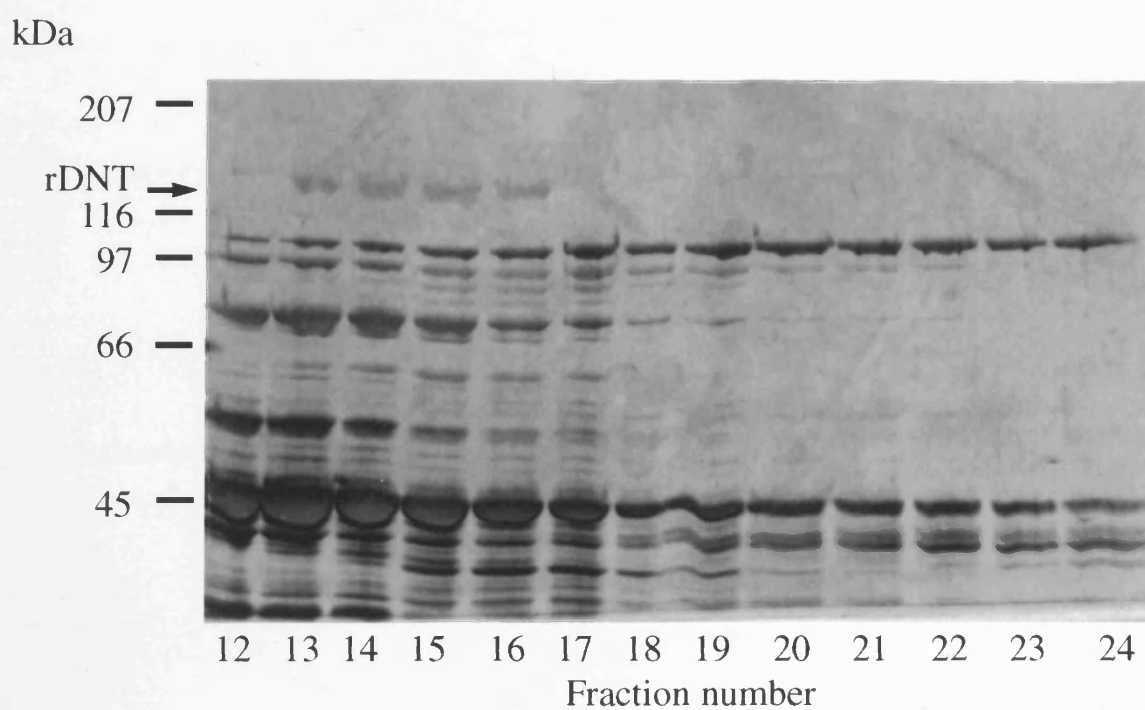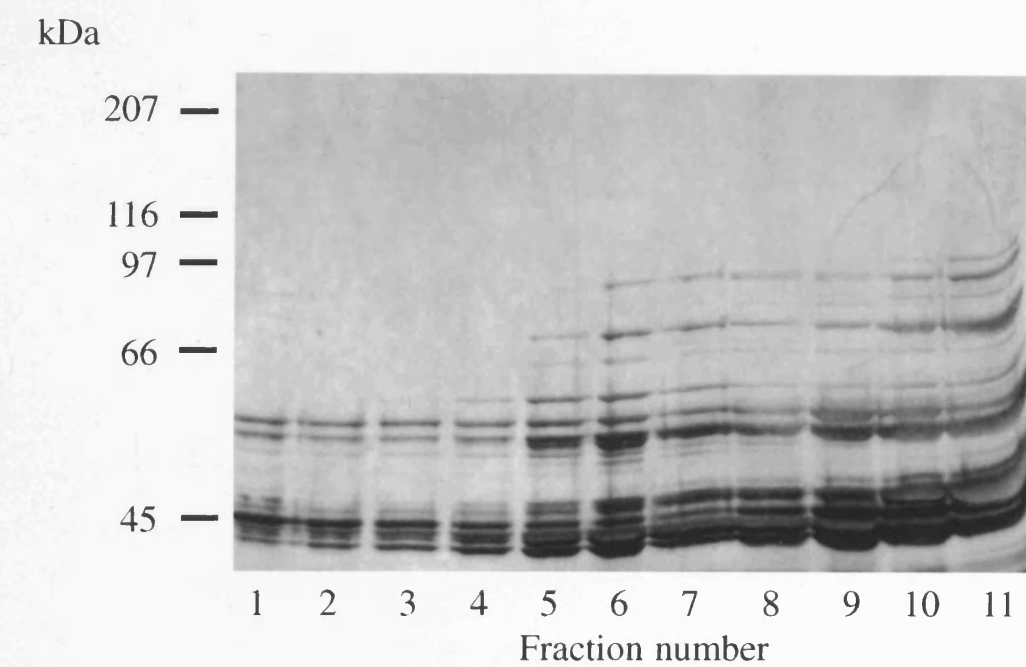
**Figure 4.9** Silver stained 8% SDS-PAGE of hydroxylapatite column fractions collected during purification of rDNT from strain DNT1. All lanes contain the same volume of sample.
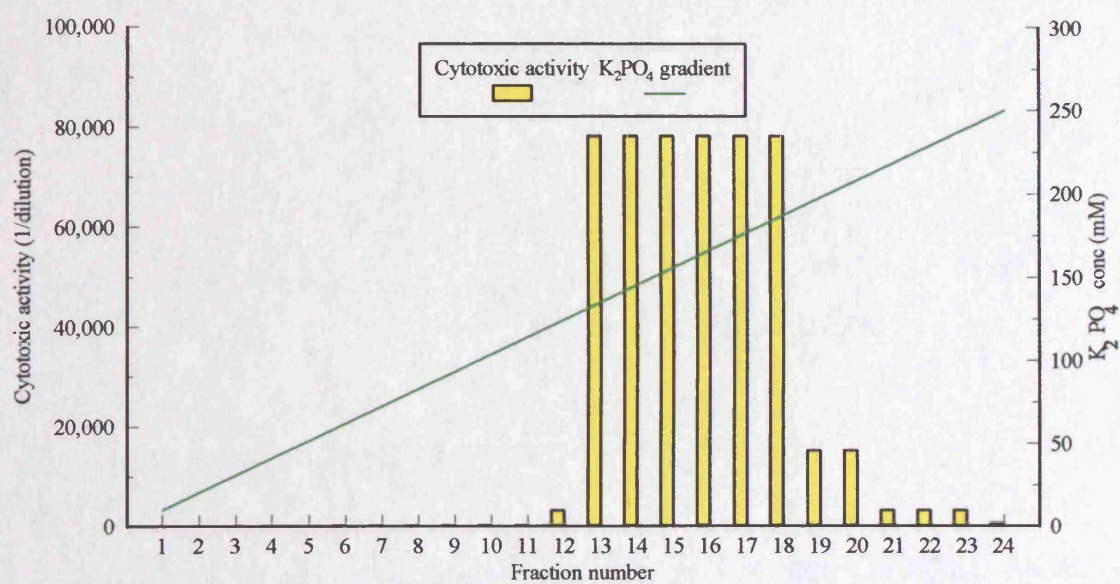
**Figure 4.10** The cytotoxic activity on EBL cells of hydroxylapatite chromatography fractions collected during the purification of rDNT from strain DNT1. All samples were tested in duplicate and diluted to an end point.

| Sample | Volume (ml) | Protein (μg/ml) | Total protein (mg) | % Protein | Cytotoxic Activity/ 20μl[d] | Total Cytotoxic Activity (U) | % Cytotoxic Activity | SA (U/mg) |
|---|---|---|---|---|---|---|---|---|
| SN[a] | 100 ml | 830 | 83 | 100 | 3125 | $1.56 \times 10^7$ | 100 | $1.9 \times 10^5$ |
| DEAE[b] | 31 ml | 560 | 17.36 | 20.9 | 15625 | $2.42 \times 10^7$ | 155 | $1.39 \times 10^6$ |
| HA[c] | 5.1 ml | 330 | 1.68 | 2.02 | 78125 | $1.99 \times 10^7$ | 127 | $1.18 \times 10^7$ |

**Table 4.1** Purification of rDNT from 300 ml culture of DNT1.

[a] Sonicate extract of DNT1.

[b] Pooled DEAE-Sephacel column fractions containing highest levels of rDNT.

[c] Pooled hydroxlyapatite column fractions containing the highest levels of rDNT.

[d] Cytotoxic activity was measured using the EBL cell assay. Cytotoxic activity was expressed as 1/endpoint-dilution, which was the last dilution of sample which was cytotoxic in over 50% of the cells. All samples were tested in duplicate. Total cytotoxic activity was adjusted for total volume of sample.

concentration of 44.8 µg ml$^{-1}$. These results are only estimates as they make the assumption that silver staining is quantitative and that all proteins stain with equal sensitivity.

### 4.2.7 Comparison of partial purification of rDNT with DNT from B. bronchiseptica

Figure 4.12 shows a SDS-PAGE analysis of equal volumes of partially purified preparations of rDNT and wild-type DNT. There are many more co-purifying proteins in the rDNT preparation compared to the wild-type DNT preparation, but the rDNT band is more intense than that of the wild-type DNT. The cytotoxic activity of the partially pure rDNT preparation on EBL cells is five times greater than the partially pure DNT preparation.

### 4.2.8 Stability of rDNT

The stability of partially pure rDNT samples was investigated by storage at 4°C and at -20°C in 50% v/v glycerol. Figure 4.13 shows that the toxin was more stable at 4°C than at -20°C. The cytotoxic activity of both toxin samples remained stable for 2 weeks, but between weeks 2 and 3 the cytotoxic activity decreased. After 25 weeks the toxin stored at 4°C was 5 times more cytotoxic than the toxin stored at -20°C.

### 4.2.9 Attempts to purify rDNT further

Several methods were investigated to try to further purify rDNT.

#### 4.2.9.1 Preparative isoelectric focusing

This technique was investigated because it was used successfully to further purify wild-type DNT after hydroxylapatite chromatography. Fractions from the hydroxylapatite column containing rDNT were pooled and dialysed against distilled water. The rDNT sample was focused in the Rotofor in the presence of 3 M urea. The initial conditions were 439 V, 23 mA and the power was limited to 12 W. It took about 4 hours 20 minutes to focus the Rotofor, and at equilibrium the values were 730 V, 14 mA and power limited. Analysis by SDS-PAGE showed a protein band of 145 kDa in Rotofor fractions 11-13, focusing with the other major proteins from the hydroxylapatite sample. These fractions, from pH 5.8 to 6.5, were cytotoxic to EBL cells. After 48 hours storage at 4°C with or without 50% glycerol, or at -20°C in 50%
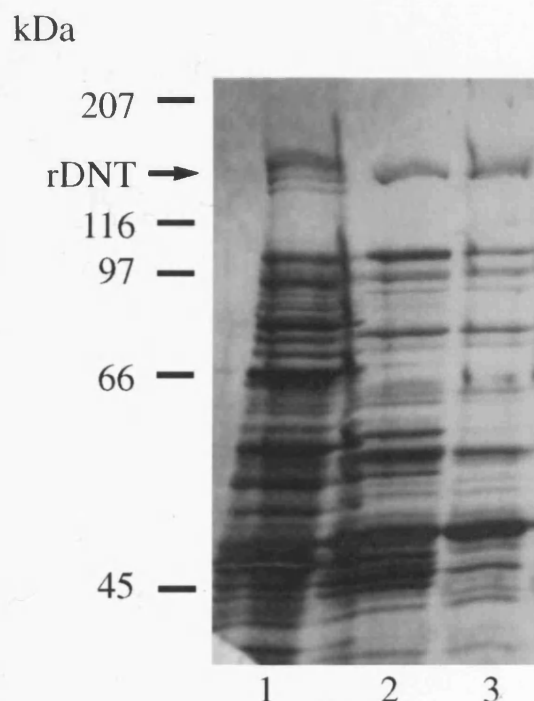
**Figure 4.11** Silver stained 8% SDS-PAGE of samples from each stage of the purification of rDNT from strain DNT1. Lane 1, sonicated cell extract of DNT1; lane 2, pooled DEAE-Sephacel column fractions containing the highest level of rDNT; lane 3, pooled hydroxyla-patite column fractions containing the highest levels of rDNT. All lanes contain the same volume of sample.
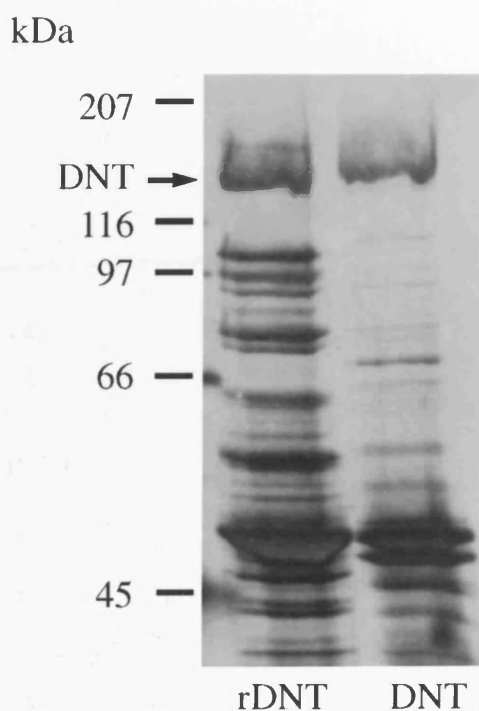


**Figure 4.12** Comparison of partially purified rDNT from strain DNT1 and partially purifed DNT from *B. bronchiseptica* by 8% SDS-PAGE. All lanes contain the same volume of sample.
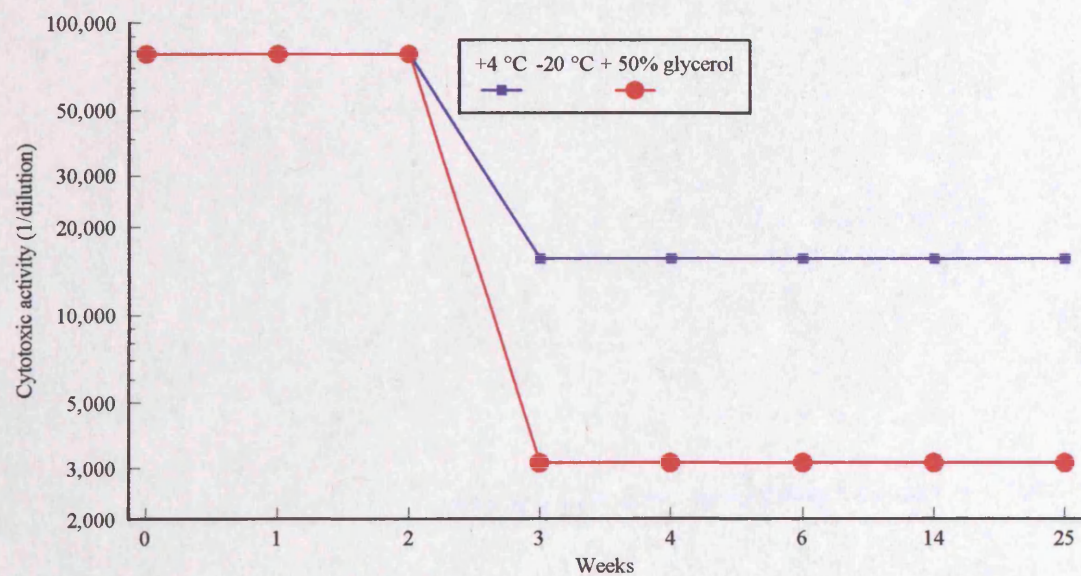
**Figure 4.13** Stability of partially pure rDNT stored at +4°C and -20°C with 50% (v/v) glycerol over a period of 25 weeks. Samples were assayed at various time points for cytotoxic activity on EBL cells. All samples were assayed in duplicate and diluted to an end point.

glycerol, the fractions that had contained rDNT showed no 145 kDa protein band by SDS-PAGE (data not shown).

### 4.2.9.2 Hydrophobic Interaction Chromatography

Hydrophobic Interaction Chromatography (HIC) was investigated because it had been successfully used in the laboratory for the purification of PMT. The HIC Media Test Kit (Pharmacia) consisted of five ready-to-use 1 ml columns, each with a different type of HIC matrix. Hydroxylapatite fractions containing rDNT were dialysed against 1 M ammonium sulphate in 50mM sodium phosphate pH 7.0, and then loaded on to the five test columns. Proteins were eluted from the columns with a stepwise descending ammonium sulphate gradient. Fractions of 1 ml were collected from the columns and analysed by SDS-PAGE and EBL cells.

Analysis of the fractions collected from the Butyl Sepharose 4 fast flow column showed that rDNT did not bind to the column and was eluted during the loading and washing of the column. None of the fractions from the Phenyl Sepharose 6 fast flow (high sub) were cytotoxic to EBL cells, and none showed a 145 kDa protein by SDS-PAGE, indicating that rDNT was not eluted from this column. On the Octyl Sepharose 4 fast flow column and the Phenyl Sepharose high performance column, rDNT was eluted in the final wash with distilled water after the decreasing ammonium sulphate gradient. On the Phenyl Sepharose 6 fast flow (low sub), rDNT was eluted from the column at 0 M ammonium sulphate in 50 mM sodium phosphate and in the final distilled water wash (data not shown).

The Phenyl Sepharose 6 fast flow (low sub) column was investigated further. The experiment was repeated using a lower salt concentration. The partially pure rDNT was dialysed against 500 mM ammonium sulphate in 50 mM sodium phosphate, pH 6.5. The column was equilibrated with this buffer, and the sample loaded and eluted with a descending stepwise ammonium sulphate gradient. The rDNT was eluted between 100 - 0 mM ammonium sulphate, and co-purified with several of the high molecular weight proteins from the partially pure sample (data not shown).

### 4.2.10 Partial purification of equivalent fraction from E.coli XL1-blue/bluescript

For subsequent experiments (Chapter 5) it was necessary to have a negative control, which differed only in DNT products. Therefore, sonicated cell extract from *E.coli* XL1 blue/bluescript was purified under exactly the same conditions as those described for strain DNT1.

The DEAE-Sephacel elution profile was similar to the elution profile obtained for the purification of rDNT from strain DNT1. SDS-PAGE analysis of the fractions (Figure 4.14) showed no 145 kDa rDNT protein band. Fractions 15-19 show a protein band of about 140 kDa, a slightly lower molecular weight than rDNT. Comparison with the SDS-PAGE from fractions of strain DNT1 showed that this 140 kDa protein is eluted just after rDNT, and was eluted with rDNT as a double band in fractions 15 and 16 (Figure 4.7). EBL cell data showed that none of the fractions from the DEAE-sephacel column was cytotoxic to the EBL cells. By comparison with SDS-PAGE analysis of fractions from DNT1, fractions 10-15 were pooled for further purification by hydroxylapatite chromatography.

The hydroxylapatite elution profile (Figure 4.15) was similar to that obtained in the purification of rDNT from DNT1, but there were more proteins in the samples. Fractions 14 -20 contained a band about 140 kDa, the protein which purifies on the DEAE-sephacel just after rDNT. None of these fractions was cytotoxic to EBL cells. The protein content was about twice that of DNT1 samples. From SDS-PAGE analysis of partially purified XL1-blue/bluescript it was decided that fraction 14 had the most similar protein profile to partially purified rDNT and was used in subsequent experiments.

### 4.2.11 Partial purification of mutant rDNT from strain K1310A

Strain K1310A is a mutant isolate of strain DNT1 in which the role of a possible nucleotide binding motif of DNT was investigated by making base substitutions in the conserved lysine residue (Pullinger *et al.*, 1996). The sonicated cell extracts from the resulting mutant, K1310A, were not cytotoxic to EBL cells, and so would provide a useful comparison with rDNT from DNT1 and XL1-blue/bluescript. Sonicated cell extract from strain K1310A was separated under exactly the same conditions as those described for strain DNT1.
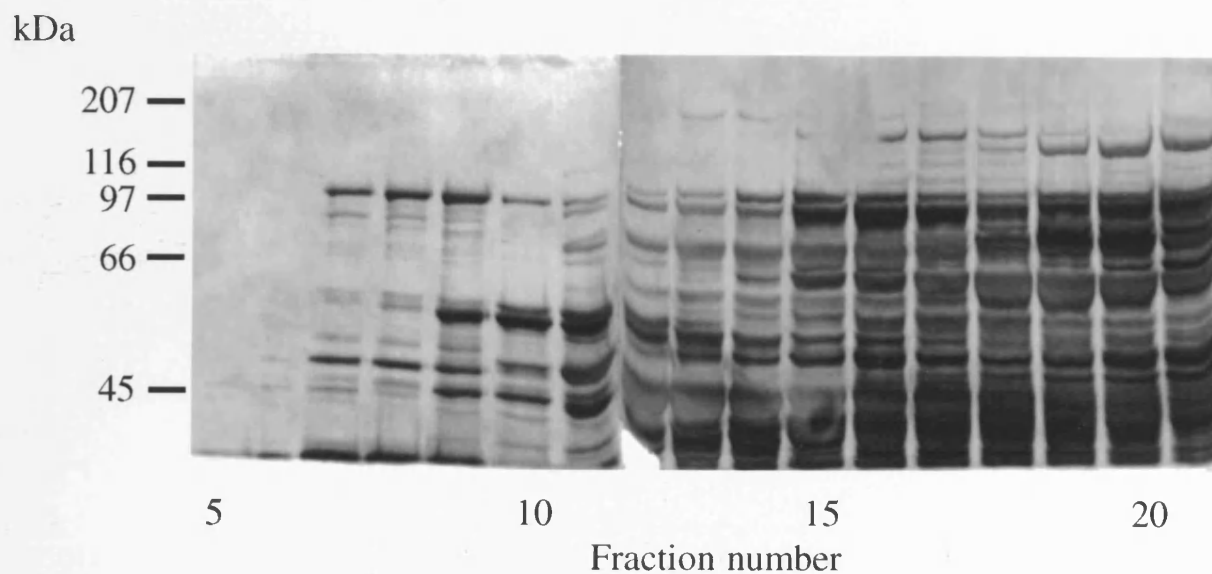
**Figure 4.14** Silver stained 8% SDS-PAGE of DEAE-Sephacel column fractions collected during purification of XL1-blue/bluescript. All lanes contain the same volume of sample.
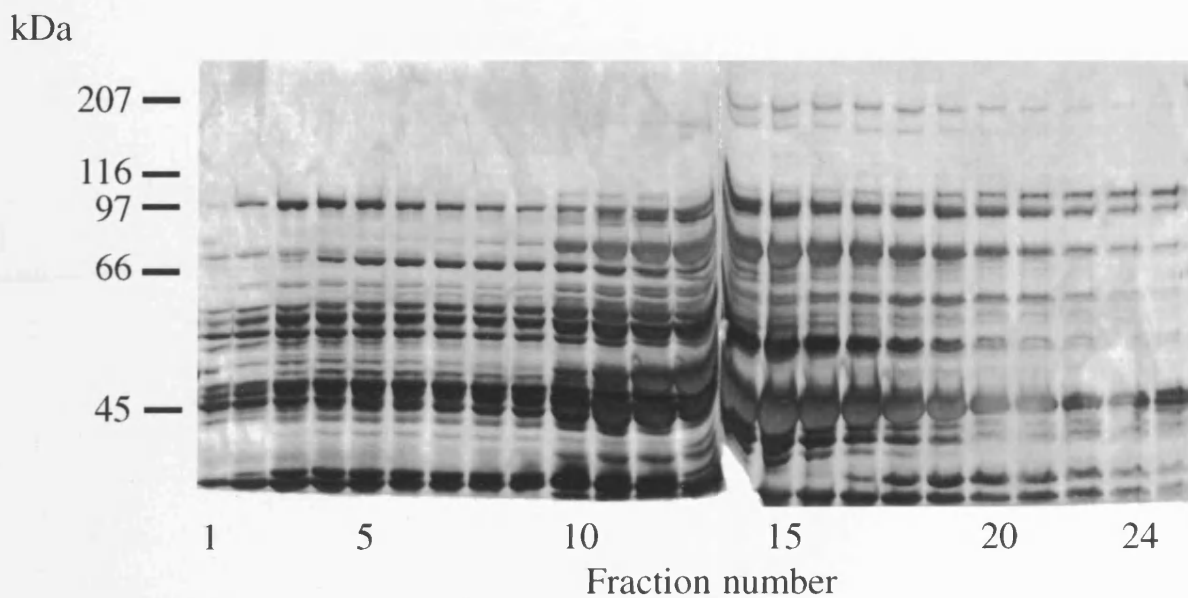


**Figure 4.15** Silver stained 8% SDS-PAGE of hydroxylapatite column fractions collected during purification of XL1-blue/bluescript. All lanes contain the same volume of sample.

The DEAE-Sephacel elution profile of sonicated cell extract from strain K1310A was similar to the elution profile obtained for the purification of rDNT from DNT1. SDS-PAGE analysis (Figure 4.16) shows a faint band in fractions 14-16 above the 140 kDa protein band which purified with rDNT. None of the DEAE-Sephacel fractions was cytotoxic to EBL cells. Based on a comparison with the protein elution profile of DNT1 it was decided to pool fractions 13-16 for further purification by hydroxylapatite chromatography.

The hydroxylapatite elution profile was similar to the elution profile obtained for the purification of rDNT from DNT1. SDS-PAGE analysis showed a protein band the size of rDNT in fractions 6-12 (Figure 4.17). This protein was eluted from the hydroxylapatite column earlier than rDNT, but all other proteins were eluted earlier too. The fractions contained more proteins than the partially pure fractions of rDNT. None of the fractions was cytotoxic to EBL cells. Previously, the cytotoxic activity of fractions had been the main criteria for choosing which fractions to pool, but, because rDNT from K1310A was not cytotoxic to EBL cells, the identity of the toxin band was confirmed by Western blot analysis. The anti-DNT monoclonal antibody AE9 (described in Chapter 6) recognised the mutant rDNT band at all stages of the purification (Figure 4.18).
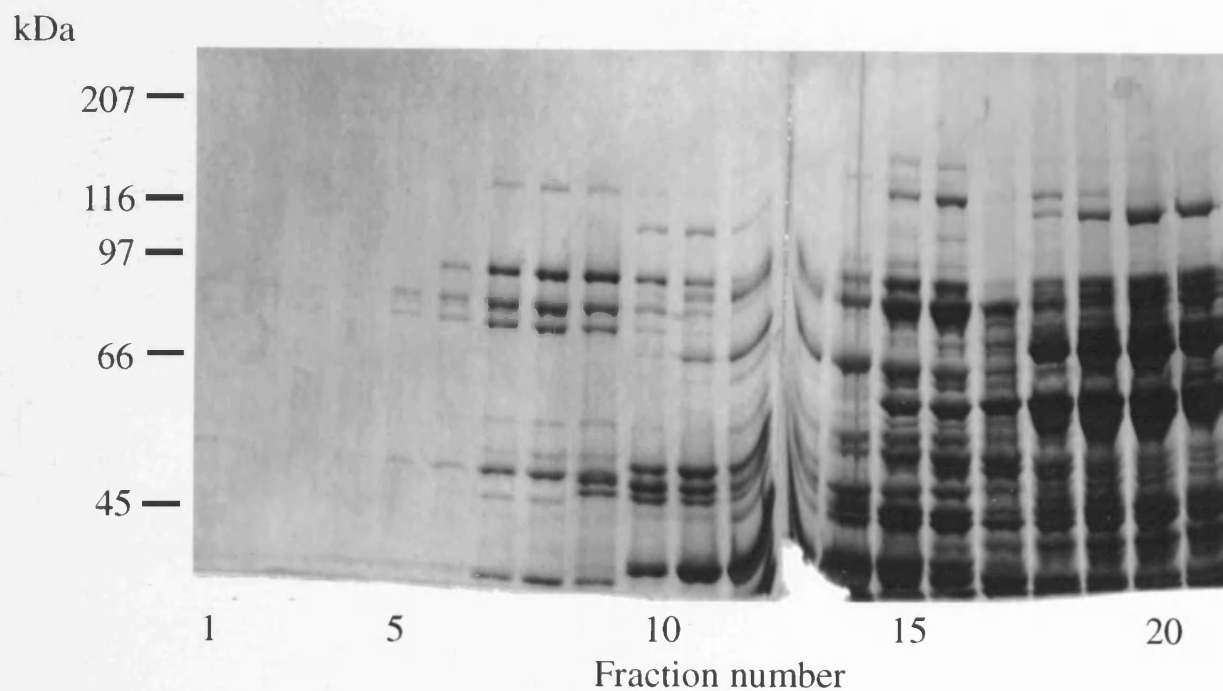
**Figure 4.16** Silver stained 8% SDS-PAGE of DEAE-Sephacel column fractions collected during purification of mutant rDNT from strain K1310A. All lanes contain the same volume of sample.
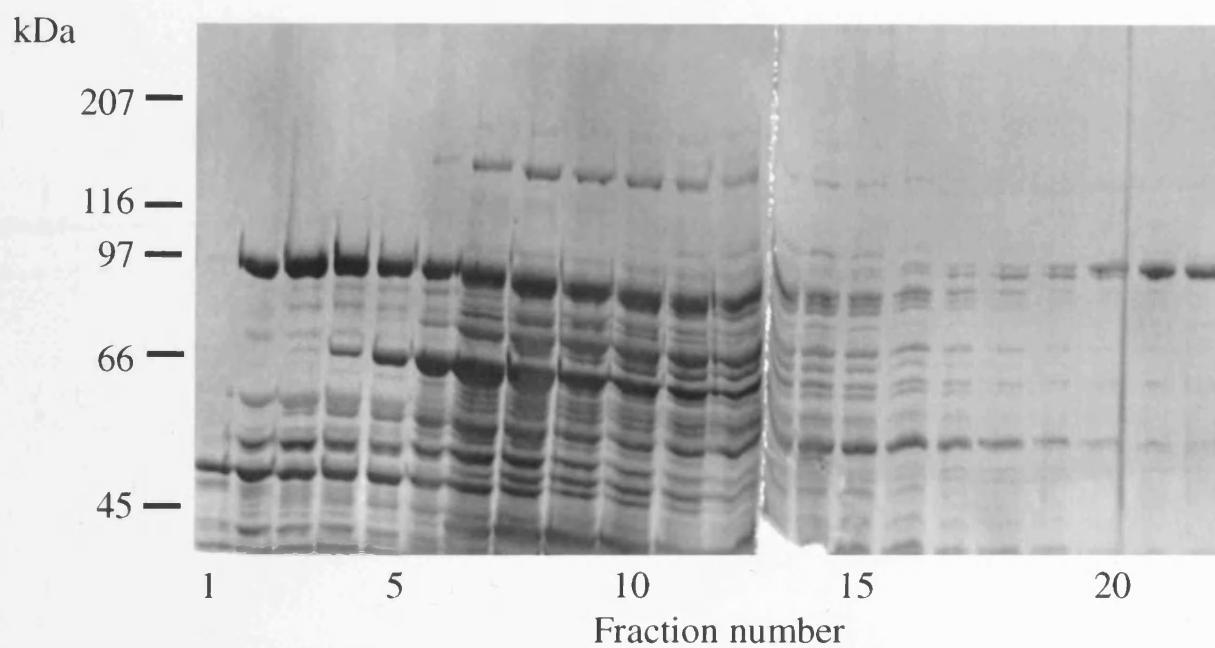


**Figure 4.17** Silver stained 8% SDS-PAGE of hydroxylapatite column fractions collected during purification of mutant rDNT from strain K1310A. All lanes contain the same volume of sample.
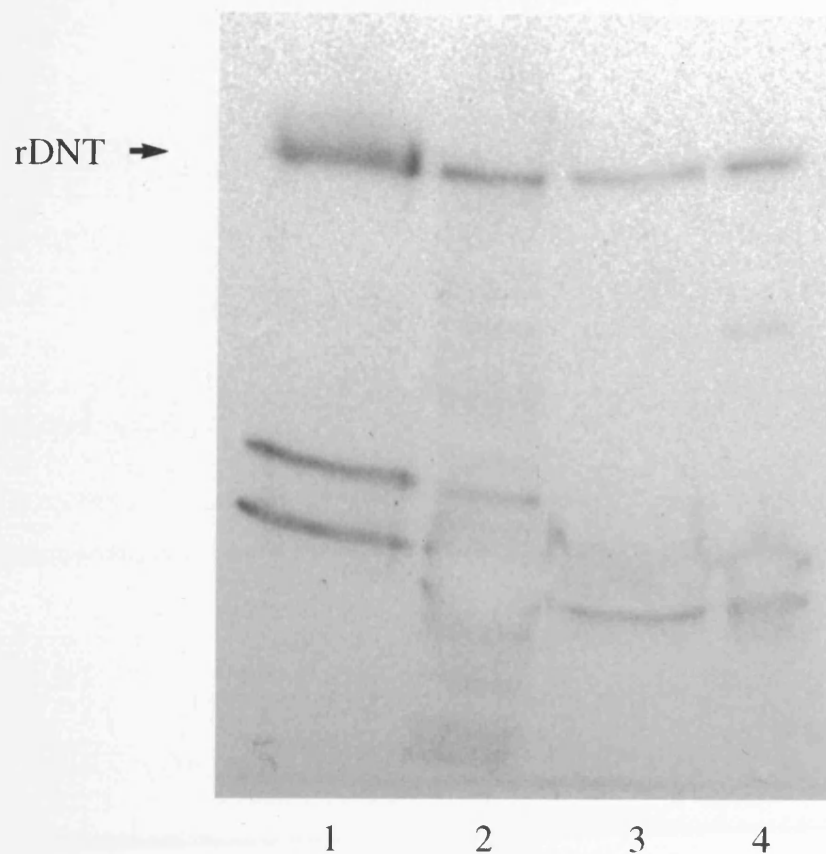
**Figure 4.18** Western blot of samples from each stage of the purification of rDNT from strain K1310A probed with anti-DNT mAb AE9.Lane 1, partially pure rDNT from DNT1; lane 2, sonicated cell extract of K1310A; lane 3, pooled DEAE-Sephacel column fractions from K1310A purification; lane 4, pooled hydroxylapatite column fractions from K1310A purification.

## 4.3 Discussion

This chapter describes the development of a small scale purification procedure for recombinant DNT from strain DNT1 (Figure 4.19). The purification procedure was based on the reproducible method developed by Garrod (1994) for the purification of wild-type DNT from *B. bronchiseptica*. A fundamental difference between sonicated cell extracts of recombinant and wild-type DNTs is that they are surrounded by different proteins and other cell components. This, in turn, could lead to different interactions of DNT with proteins, and differences in separation. However, since the methods of Garrod (1994) were successful in separating DNT from *B. bronchiseptica* cell extracts, this method was adopted as the basis for purification of rDNT.

The data shown for the purification of rDNT from DNT1 was representative of five separate purification runs performed using the final protocol. Fractions containing rDNT were identified by the presence of a 145 kDa protein by SDS-PAGE, and correlation with a peak in cytotoxicity on EBL cells. Final preparations contained rDNT at a concentration of approximately 33 $\mu$g ml$^{-1}$, but contained several major co-purifying proteins. During the development of the purification procedure for rDNT there had been confusion with a protein of similar size to rDNT which eluted from the DEAE-Sephacel column with or just after rDNT. The DEAE-Sephacel chromatography of XL1-blue/bluescript showed that this protein is an *E. coli* protein eluted just after the position in which rDNT was expected to be eluted.

Initially the growth of DNT1 and the production of rDNT from the clone was investigated. The pattern of rDNT production was similar to that of wild-type DNT from *B. bronchiseptica* grown in Cohen-Wheeler broth. DNT production increased in parallel with the increase in cell numbers during the log phase of growth, and decreased with the decrease in cell numbers during cell death (Nakai *et al.*, 1985). Similar results have been shown for the production of DNT from *B. pertussis*, for example Cowell *et al.* (1979) found that *B. pertussis* cells grown in modified Stainer and Schölte medium were most toxic during the mid-log phase of growth.

In LB broth, the decrease in cytotoxic activity of rDNT during death phase could not have been due to degradation of the toxin by proteases released by lysed or leaking bacteria, because the rDNT protein band was visible by SDS-PAGE after 24 hours growth. Recombinant DNT may have aggregated to other proteins or cell

DNT1

Enzymatic
lysis

Sonication

DNAase and RNAase
treatment

DEAE-Sephacel
Chromatography
linear ionic gradient

DEAE-Sephacel
Chromatography
step-wise ionic gradient

Hydroxylapapitite
chromatography

Preparative isoelectric
focusing
plus 3M urea

Hydrophobic
interaction
chromatography
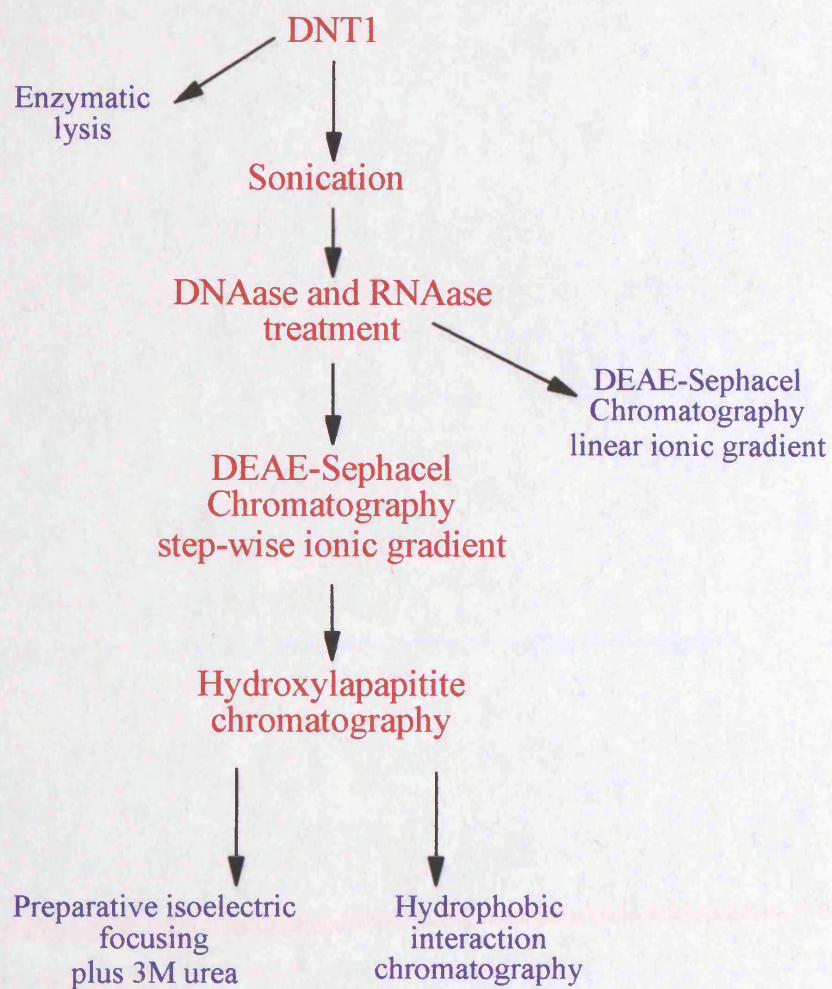
**Figure 4.19** Purification procedure for rDNT. Successful purification steps in red, and unsuccessful purification steps in blue.

components causing loss of activity but was separated by SDS-PAGE. The rDNT cytotoxic activity was stable for 24 hours when DNT1 was grown in 2 × YT medium, even though the bacterial culture was in death phase. A component of 2 × YT medium may have stabilised the toxin.

These experiments with the two different media showed that there was little difference in the growth of DNT1, and the amount of rDNT produced in LB broth or 2 × YT broth. However, it was decided to used 2 × YT medium for the growth of DNT1 in subsequent experiments, because the rDNT produced was more stable.

Recombinant DNT was shown to be an intracellular protein, like wild-type DNT, and had to be released from the bacterial cells for purification. Lysis of the bacterial cells with lysozyme lysis buffer was effective in releasing active rDNT. Dialysis of the cell lysate against the ion-exchange column buffer resulted in the loss of rDNT from the sample. Dialysis of the cell lysate from high to low salt concentration resulted in the precipitation of proteins. Since DNT is reported to be insoluble at low ionic strength (Endoh *et al.*, 1986c) it is likely to have precipitated out, and this would explain why it could not be visualised by SDS-PAGE and why no cytotoxic activity was observed. This was confirmed when the cell lysate was dialysed against a buffer containing 50 mM NaCl, half the concentration of salt in the lysis buffer, and there was no precipitation of proteins and rDNT was not lost. Unfortunately, the NaCl concentration was too close to that required for the elution of rDNT from the DEAE-Sephacel column, and when the cell lysate was loaded on to the column, rDNT did not bind. Attempts to lyse DNT1 with buffers containing either no NaCl or 10 mM NaCl resulted in effective lysis, but none of the extracts contained rDNT.

Due to the problems of enzymatic lysis of *E. coli* DNT1 cells, sonic disruption was investigated, but only after modifications were made to improve safety. A simple experiment showed the optimum sonication conditions to produce a cell-free extract which contained active rDNT. Before the sonicated cell extract was removed from the category 3+ laboratory it had to be free of nucleic acid and so it was treated with DNAase and RNAase. This process had no effect on rDNT.

When a linear ionic gradient was used to elute proteins from the DEAE-Sephacel column, rDNT was eluted in all the fractions. This poor resolution could be

due to many factors: aggregation of the toxin to other proteins, precipitation of toxin on the column, too much mass loaded onto the column, or the gradient too steep. A stepwise ionic gradient was used to elute proteins from the column because a stepwise elution has the potential for higher resolution. Recombinant DNT was eluted from the column at 150-180 mM NaCl, approximately the same concentration of NaCl used to elute wild-type DNT. The fractions containing rDNT were pooled for further purification.

The hydroxylapatite column successfully separated proteins in the pooled fractions from the DEAE-Sephacel column. Recombinant DNT was eluted from the column at approximately 180 mM phosphate buffer, the same concentration of phosphate buffer used to elute wild-type DNT from the column. Fractions collected from the hydroxylapatite column which contained rDNT were referred to as partially pure preparations of rDNT.

The partially pure rDNT preparations were not as pure as the wild-type DNT preparations produced using a similar purification procedure. This method resulted in a 62-fold purification of rDNT from DNT1, whereas, the similar method resulted in a 104-263-fold purification of DNT from *B. bronchiseptica*. This difference in level of purification was due to the toxin being separated from a different mixture of proteins in the *B. bronchiseptica* and *E. coli* sonicated cell extracts. Published methods give an increase in purification of *Bordetella* DNT between 89- (Horiguchi *et al.*, 1989) and 1,350- fold (Zhang and Sekura, 1991). My purification of rDNT gave a lower fold of purification compared to other methods, due to the different co-purifying proteins. However, the specific activity of the partially pure rDNT was $1.18 \times 10^7$ U activity/mg protein, higher than the $1.6 \times 10^6$ U/mg determined by Horiguchi *et al.* (1989), and lower than $2.7 \times 10^9$ U/mg determined for the pure DNT of Zhang and Sekura (1991). All the published methods used the suckling mouse model to detect the activity of DNT. The EBL cell assay has been shown to reflect the results obtained using the suckling mouse model (Garrod, 1994). However, Pullinger *et al.* (1996) found that the difference in activity between DNT1 and B58 sonicated cell extracts using the suckling mouse model was not as great as expected from the difference in cytotoxicity on EBL cells. This suggested that the two assays do not respond quantitatively. Therefore, the specific activities of preparations from this study and

published methods will vary due to the different assays used to measure the activity of DNT. The rDNT total activity increased during the purification proceedure. It is possible that other cell components in the sonicated cell extract interacted with rDNT inhibiting its activity or interfered with the cell assay, and as these components were removed during purification the activity of rDNT increased.

The storage experiments show that rDNT, like wild-type DNT, is an unstable protein. The partially pure rDNT samples were more stable at 4°C than at -20°C, contrary to the results for wild-type DNT, which was more stable at -20°C than 4°C (Garrod, 1994). However, wild-type DNT was more stable stored at -20°C without glycerol than with glycerol, and in my storage experiments rDNT was stored at -20°C only in the presence of glycerol, so the findings are not directly comparable. Horiguchi et al. (1990) reported that purified DNT was unstable at -20°C and -80°C. Partially pure rDNT was stored at 4°C for short periods and for experiments in which activity was crucial, freshly purified rDNT was used.

Several other techniques were investigated in an attempt to further purify rDNT. The pooled hydroxylapatite fractions containing rDNT were focused on the Rotofor using the same conditions developed for wild-type DNT. Recombinant DNT was focused at pH 5.8-6.5, about the same pH at which wild-type DNT focused. Unfortunately, the major contaminating proteins from the hydroxylapatite fractions also focused at this pH with rDNT. It may have been possible to remove these proteins by refractionating the fractions or using a narrower pH gradient. However, rDNT was lost after 48 hours storage at 4°C, so the same problems of storage would apply to rDNT as to the wild-type DNT, and this method was not further investigated.

Hydrophobic interaction chromatography (HIC) exploits differential hydrophobic interactions between an adsorbent and proteins as a means of separation. The simplest model of a protein is a hydrophilic shell surrounding a hydrophobic core. But hydrophobic domains do occur on the surface of the protein due to the presence of side chains from non-polar amino acids such as alanine, methionine, tryptophan and phenylalanine. The number, size and distribution of these non-ionic regions is characteristic to each protein and can be used as a basis for separation. Generally, hydrophobic interactions are increased with an increase in ionic strength. The salting-out ions decrease the availability of water molecules in solution, increasing surface

tension and enhancing hydrophobic interactions. In HIC, proteins interact with a non-ionic group (e.g. octyl/phenyl) attached to an inert matrix, in the presence of salting-out ions such as sodium chloride or ammonium sulphate. Hydrophobic interactions are reduced by lowering the concentration of salting-out ions, and proteins are eluted from the column. The HIC Media Test Kit (Pharmacia) was used to investigate which hydrophobic matrix was the best for purification of rDNT. There are many different types of hydrophobic matrix, each differing in their hydrophobicity. For example, Phenyl Sepharose is less hydrophobic than Octyl Sepharose, and therefore extremely hydrophobic proteins may adsorb too strongly to Octyl Sepharose requiring strong eluting conditions. The hydrophobicity of the column can also be changed by the level of coupling of the adsorbent to the matrix e.g. Phenyl Sepharose 6 fast flow low sub and high sub columns.

Recombinant DNT bound very strongly to all the adsorbents tested, except Butyl Sepharose. It was eluted from all the columns near the end of the salt gradient or in the final water wash, with the exception of the Phenyl Sepharose 6 fast flow (high sub) where rDNT remained bound to the column. Phenyl Sepharose 6 fast flow (low sub) was further investigated because rDNT was eluted from the column in the final salt steps of the gradient, and all fractions contained few other proteins. The starting salt concentration was reduced to weaken the strength of binding so that rDNT was eluted earlier from the column, and the pH was lowered nearer the pI of rDNT. Recombinant DNT was eluted from the column earlier but over a wide ionic strength range. The major contaminating proteins from the hydroxylapatite fractions co-purified with rDNT, and so HIC was not further investigated.

XL1-blue/bluescript was purified in exactly the same way as DNT1 to obtain a partially purified fraction which contained the same *E. coli* proteins as in the partially pure rDNT, except for rDNT. Fraction 14 from the hydroxylapatite chromatography of XL1-blue/bluescript contained all the major *E. coli* proteins that were in the partially pure rDNT fractions, and so it was used in subsequent experiments as a comparison with rDNT.

Non-cytotoxic mutant rDNT from strain K1310A was purified in exactly the same way as DNT1 to allow further investigation of the mutant toxin and to compare it to rDNT. An anti-DNT monoclonal antibody recognised a 145 kDa protein from

each step of the purification procedure, confirming that this protein was the mutated rDNT toxin. Fraction 8 from the hydroxylapatite column had the most similar protein profile to that of partially pure rDNT preparations, and was used in subsequent experiments.

The purification method developed as part of this thesis proved to be quick, reliable and resulted in partially pure preparations of rDNT with high cytotoxic activity. Unfortunately, pure preparations of rDNT were not obtained because the length of the time involved in separating out co-purifying proteins would have limited the time investigating the biological activity of the toxin, especially when it is considered that storage time of rDNT is a limiting factor. Although, preparations of rDNT were not as pure as preparations obtained from wild-type DNT using similar purification methods, the recombinant toxin had the distinct advantage that good negative controls could be used, namely XL1-blue/bluescript fraction 14 and K1310A. Therefore, partially pure rDNT was used in subsequent experiments to characterise DNT.

# Chapter 5

## Biological effects of partially pure recombinant DNT

## 5.1 Introduction

DNT is so named because of its ability to produce dermonecrotic lesions when injected subcutaneously into experimental animals. These lesions are species-dependent, causing ischaemic lesions in rabbits and guinea pigs, and haemorrhagic lesions in suckling mice. The effect in mice is age-dependant, and mice over seven weeks old are unresponsive to DNT (Parton, 1986). Pullinger *et al.* (1996) have shown that crude lysates of rDNT are dermonecrotic in the suckling mouse model, producing similar lesions to those produced by wild type DNT.

The effect of DNT on a number of tissue culture cell lines has been studied. Endoh *et al.* (1988a; 1988b) and Nagai *et al.* (1992a) showed that DNT acted specifically on smooth muscle cells, but had no effect on cardiac or skeletal muscle cells. Embryonic bovine lung (EBL) cells have been used by several groups to assay for *B. bronchiseptica* DNT (Kume *et al.*, 1986; Magyar *et al.*, 1988; Garrod, 1994). The EBL cell assay was well established at this Institute, and originally used for assaying toxigenic *P. multocida* (Rutter and Luther, 1984), and Garrod (1994) used this method to assay partially purified DNT. *B. pertussis* DNT has also been shown to act on human embryonic lung cells (Frampton *et al.*, 1991).

Garrod (1994) showed that partially pure DNT stimulated DNA synthesis in quiescent Swiss 3T3 cells. Swiss 3T3 cells are cultured murine fibroblasts, and used as a model system for the study of signal transduction pathways. The cells cease to proliferate in the $G_0$ phase of the cell cycle when they deplete the medium of its growth promoting activity and enter a quiescent or non-dividing state. The quiescent cells can be stimulated to reinitiate DNA synthesis and cell division by replenishing the medium with fresh serum or adding growth factors, neuropeptides, or pharmacological agents in serum-free medium (Rozengurt, 1986). DNA synthesis can be measured by the incorporation of tritiated thymidine into the acid insoluble fraction of the cells (Dicker and Rozengurt, 1978). Labelled deoxythymidine is converted to deoxythymidine 5'-triphosphate (dTTP), via deoxythymidine monophosphate (dTMP), and incorporated into DNA. The cells arrest in $G_2$ of the cell cycle, and cell division is inhibited due to the presence of tritiated thymidine. Therefore, cell numbers remain constant, but the amount of DNA per cell increases, and so DNA synthesis is directly proportional to the thymidine incorporation and the number of cells stimulated.

Horiguchi *et al.* (1995) showed that DNT could induce the formation of actin stress fibres and focal adhesions in osteoblast like cells. Stress fibres are contractile bundles of actin filament, and the sites where stress fibres are linked via integrins to the extracelluar matrix are known as focal adhesions. Regulation of stress fibre and focal adhesion formation is controlled by a small GTP-binding protein, Rho (Hall, 1994).

The Rho family belongs to a superfamily of Ras-related GTP-binding proteins. The Rho family can be divided into three subfamilies Rho, Rac and Cdc42, all of which are involved in regulating the organisation of the actin cytoskeleton (Hall, 1994). The Rho subfamily consists of RhoA, -B and -C, which are 85% identical but appear to have different cellular locations (Adamson *et al.*, 1992). RhoA regulates the formation of actin stress fibres and focal adhesions (Ridley and Hall, 1992), and is implicated in a variety of cellular activities including cell motility (Takaishi *et al.*, 1993), cell cycle progression (Yamamoto *et al.*, 1993; Aepfelbacher *et al.*, 1995), and the formation of actin contractile ring during cytokinesis (Kishi *et al.*, 1993; Mabuchi *et al.*, 1993). RhoB expression varies during the cell cycle and affects cell transformation (Zalcman *et al.*, 1995). Rac regulates lamellipodium formation and membrane ruffling, while Cdc42 regulates filopodium formation (Nobes and Hall, 1995). Rac and Cdc42 regulate the formation of focal complex structures associated with lamellipodia and filopodia (Nobes and Hall, 1995). Rho family proteins may be involved in regulation of cell growth and mitogenesis (Ridley, 1996; Symons, 1996). It has been reported that microinjection of quiescent Swiss 3T3 cells with Rho, Rac or Cdc42 stimulated cell cycle progression through $G_1$ and subsequent DNA synthesis (Olson *et al.*, 1995).

Rho GTP-binding proteins cycle between an active GTP-bound form and an inactive GDP-bound form (Hall, 1994). This cycle is regulated by guanine-nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine-nucleotide dissociation inhibitors (GDIs) (Takai *et al.*, 1995). This allows Rho proteins to behave as molecular switches and regulate many intracellular signalling pathways. Members of the Rho and Ras subfamilies are organised in cascades activating multiple pathways. These pathways included the mitogen-activated protein kinase (MAPK) cascades, which activate transcription, and other pathways involved in cytoskeleton

reorganisation (Figure 5.1). The Ras signalling pathways are activated by a ligand binding to a specific receptor tyrosine kinase (RTK). Ras is known to activate the classic MAPK cascade via Raf kinase and extracellular-regulated kinase (ERK) leading to cell proliferation (Moodie and Wolfman, 1994). The formation of stress fibres and lamellopodia induced by Ras are dependent on Rho and Rac respectively (Ridley *et al.*, 1992). Rho and Rac are essential for transformation of fibroblast cells by oncogenic Ras (Qiu *et al.*, 1995). Both these results suggest that Rho and Rac can act downstream of Ras, and that Rac functions upstream of Rho. Rac is also activated by Cdc42 (Nobes and Hall, 1995). There has been a lot of research on the role of Rac and Cdc42 in the activation of kinase cascades that could potentially be involved in mitogenic responses. Rac and Cdc42 bind to and active the 21p-associated kinase (PAK) family of serine/threonine kinases. In turn PAK activates the Jun amino-terminal kinase (JNK) and p38 MAPK cascade, which could lead to activated transcription by serum response factor (SRF) and cell proliferation (Vojtek and Cooper, 1995; Symons, 1996). Less is known about the Rho cascades involved in cell proliferation. Rho actives SRF through a pathway independent from either the Cdc42/Rac or Ras pathways. Rho might activate an as yet unidentified MAPK cascade which regulates transcription (Ridley, 1996; Symons, 1996).

Although the precise molecular pathway of the activation of Rho proteins and the downstream events leading to cytoskeletal rearrangements are not fully understood, there is much research in this field (Takai *et al.*, 1995; Machesky and Hall, 1996; Ridley, 1996). Recently, a new model has been suggested for RhoA induced stress fibre formation involving the phosphorylation of the myosin light-chain (MLC), thereby increasing the interaction of myosin with actin filaments (Figure 5.2) (Ridley, 1996). Two groups, Leung *et al.* (1995; 1996) and Matsui *et al.* (1996) have shown that a Rho-kinase, ROKα, is a key component of the RhoA and actin cytoskeleton pathway. ROKα stimulates the formation of stress fibres and focal adhesions when over-expressed in HeLa cells (Leung *et al.*, 1996). RhoA can stimulate the kinase activity of ROKα *in vitro* (Leung *et al.*, 1995; Matsui *et al.*, 1996), suggesting that ROKα lies downstream of RhoA in the pathway leading to stress fibre formation. Kimura *et al.* (1996) showed that RhoA binds to the myosin-binding subunit (MBS) of myosin phosphatase, which regulates phosphorylation of MLC. ROKα
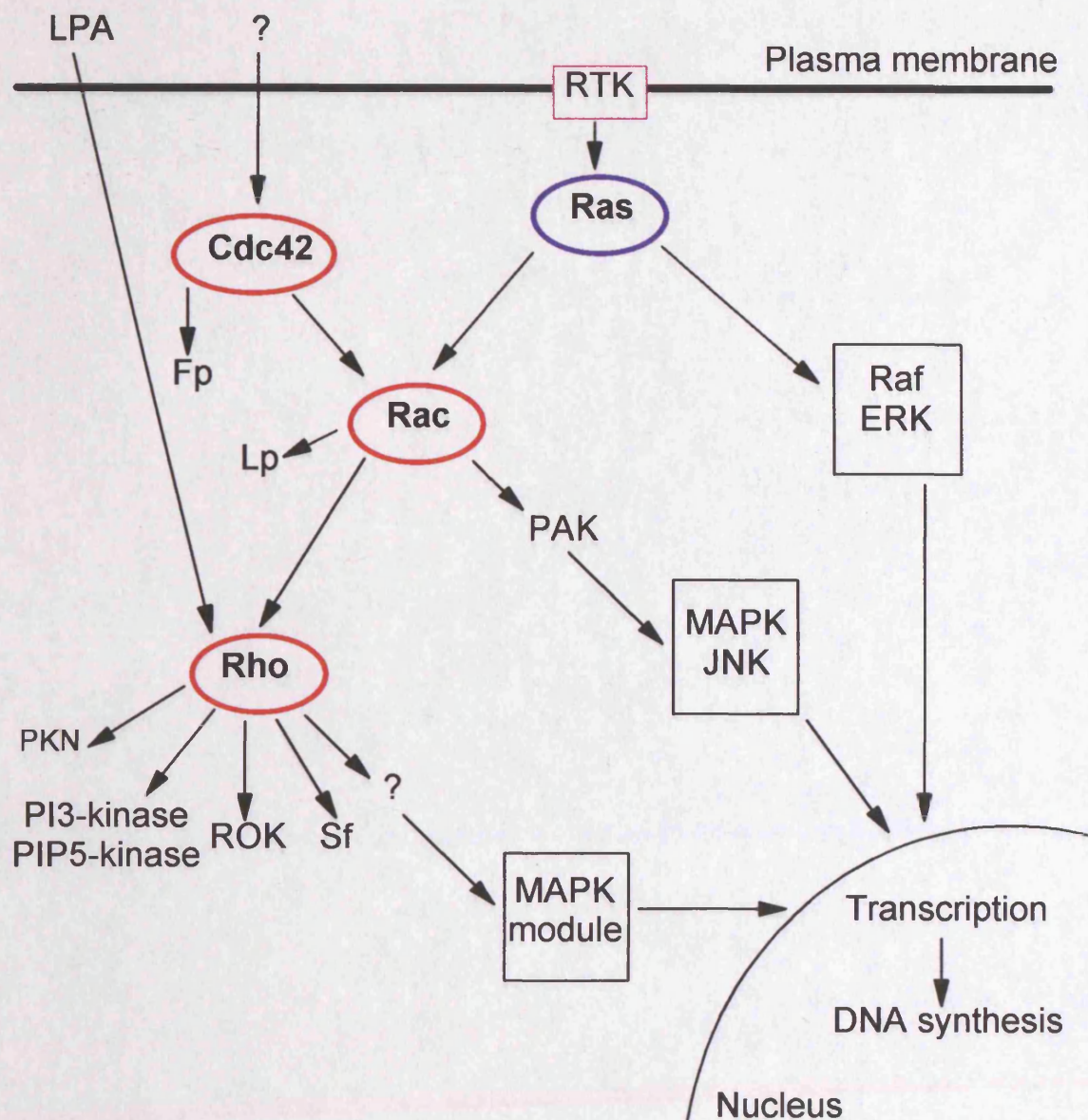
**Figure 5.1** Cascade organization of the Rho family GTP-binding proteins.
Ras is activated by a ligand binding to a specific receptor tyrosine kinase (RTK). Ras stimulates both the Rac and extracellular-signal regulated kinase (ERK). Cdc42 regulates multiple pathways including: filipodia formation (Fp), Rac activation, and stimulation of the mitogen-activated protein kinase (MAPK) and the Jun amino-terminal kinase (JNK) pathways. Rac stimulates lamellipodia formation (Lp), activation of Rho, and activation of the MAPK and JNK pathways through activation of p21-associated kinases (PAK). Rho is also activated by lysophosphatidic acid (LPA). Rho induces stress fibre formation (Sf), activation of PI3-kinase, PIP5-kinase, the Rho-binding kinase (ROK), protein kinase N (PKN), and a unidentified MAPK module. Adapted from Symons (1996).
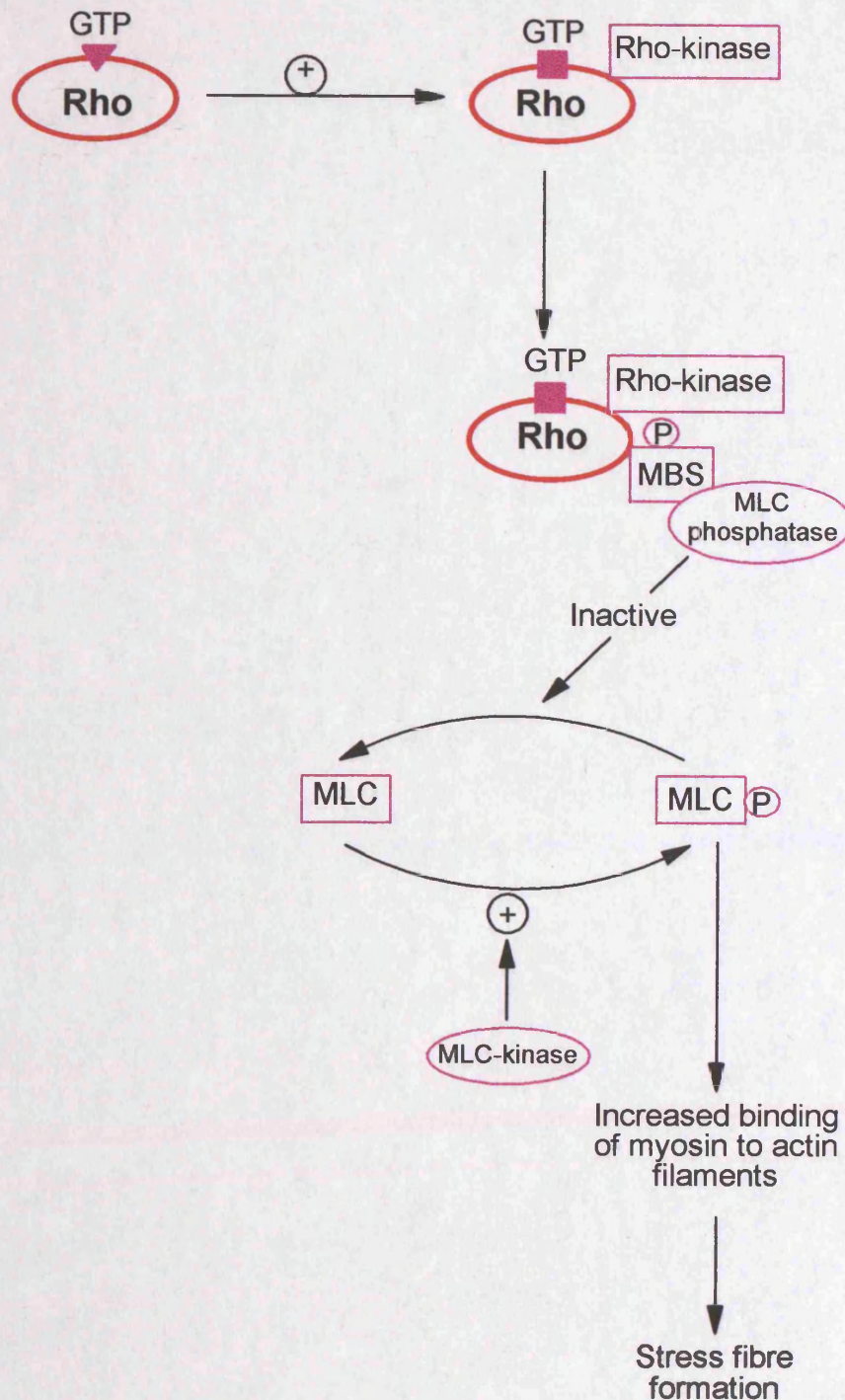
**Figure 5.2** Model for Rho-induced stress fibre formation.
Activated Rho interacts with Rho-kinase and with myosin-binding subunit (MBS) of myosin light chain (MLC) phosphatase. Active Rho-kinase phosphorylates MBS, leading to inactivation of the MLC phosphatase. Phosphorylated MLC accumulates leading to increased binding of myosin to actin filaments and formation of stress fibres. Adapted from Ridley (1996).

phosphorylates MBS (Matsui *et al.*, 1996) and inhibits the myosin phosphatase activity. This results in accumulation of phosphorylated MLC by MLC-kinase. Phosphorylation of MLC induces conformational changes in myosin II which increases the interaction with actin and formation of myosin filaments along actin stress fibres (Tan *et al.*, 1992).

These are not the only downstream targets of Rho. Due to the diverse downstream effects of Rho many targets of Rho have been identified. A protein kinase N (PKN), which is a ser/thr protein kinase, binds to and is activated by RhoA, but the substrates of PKN are unknown (Amano *et al.*, 1996; Watanabe *et al.*, 1996). RhoA has also been implicated in the modulating phospholipases and phosphoinositide kinases, including phosphatidylinositol 4-phosphate 5-kinase (PIP5-kinase) (Chong *et al.*, 1994) and phosphatidyl inositol 3-kinase (PI3-kinase) (Kumagai *et al.*, 1993).

RhoA induces focal adhesion formation and stimulates tyrosine phosphorylation of focal adhesion proteins, including pp125[FAK], p130 and paxillin (Flinn and Ridley, 1996). No tyrosine kinase has been identified which directly interacts with Rho, therefore the Rho induced protein tyrosine phosphorylation may be indirect and mediated by other Rho targets. Tyrosine phosphorylation of paxillin, pp125[FAK] and p130 in response to Rho was observed under conditions when actin stress fibre formation was inhibited (Flinn and Ridley, 1996). This suggests that stress fibre formation and focal adhesion formation are separate and therefore regulated by independent Rho-induced downstream events (Machesky and Hall, 1996).

The Rho family is a very important group of signalling proteins that regulate the cytoskeleton and are capable of interacting with other proteins to regulate various cell functions. However, much more research is required before the multiple signalling pathways are fully understood.

The aim of this work was to investigate the biological activity of rDNT. Horiguchi *et al.* (1995) suggested that DNT directly modified the small GTP-binding protein, Rho. Work in our laboratory has also shown that crude lysates of rDNT can directly modify recombinant RhoA *in vitro*, but neither Rac1 nor Cdc42 was modified (G. Pullinger, personal communication). Furthermore, crude lysates from the mutant strain K1310A was inactive in this assay. To investigate whether Rho that had been affected by rDNT could induce regulation of the cell cytoskeleton, I studied the effects

of partially pure rDNT on Swiss 3T3 cell morphology, actin stress fibre formation and focal adhesion formation. I also investigated *in vivo* rDNT modification of Rho by screening Western blots of crude lysates of rDNT-treated cells with an anti-RhoA monoclonal antibody. In this work I investigated whether rDNT could stimulate DNA synthesis in quiescent Swiss 3T3 cells, and studied the uptake of the toxin into the cells. Partially purified proteins from XL1-blue/bluescript and purified mutant rDNT from K1310A were used as controls to show conclusively that DNT was responsible for biological activities previously observed using partially pure wild-type DNT.

## 5.2 Results

### 5.2.1 Morphological changes in cells treated with rDNT

Figure 5.3 shows the morphological changes in EBL cells when treated with rDNT. Control wells, which contained EBL cells and media only, formed a confluent monolayer of elongated, thin cells which gave an overall swirling appearance. Cells which had been treated with rDNT samples were more rounded with darkly stained nuclei and the monolayer was disrupted with gaps. The same morphological changes were described in EBL cells treated with partially pure wild-type DNT (Garrod, 1994). A 20 μl sample of partially pure rDNT at an estimated concentration of 30 μg $ml^{-1}$ was cytotoxic to a dilution of 1 in 78,125. This gave a minimum cytotoxic dose of 7.68 pg $ml^{-1}$. When partially pure rDNT was heated to 56°C for 30 minutes and assayed on EBL cells these morphological changes were not seen.

Figure 5.4 shows the morphological changes in quiescent Swiss 3T3 fibroblast cells under serum-starved conditions after treatment with rDNT. Many of the cells treated with rDNT were enlarged and binucleated. These changes were the same as the morphological changes induced by wild-type DNT (Garrod, 1994). Recombinant DNT induced morphological changes in Swiss 3T3 cells in a dose-dependant manner. Figure 5.5 shows the appearance of binucleated cells in Swiss 3T3 cells under serum-starved conditions after treatment with various concentrations of rDNT for 24 hours. The concentration of rDNT was determined as described in Section 4.2.6. Recombinant DNT at 40 ng $ml^{-1}$ caused 31.3% binucleation of Swiss 3T3 cells, and in control cells, not treated with rDNT, only 1.4% of the cells were binucleated.

A
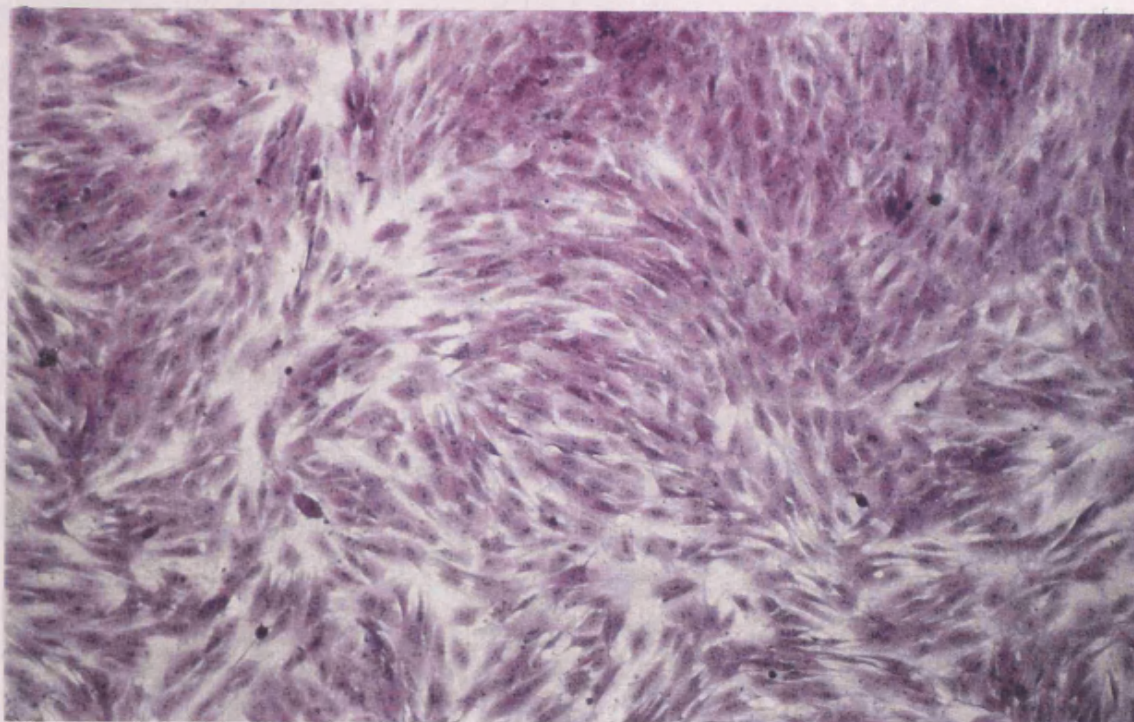


B
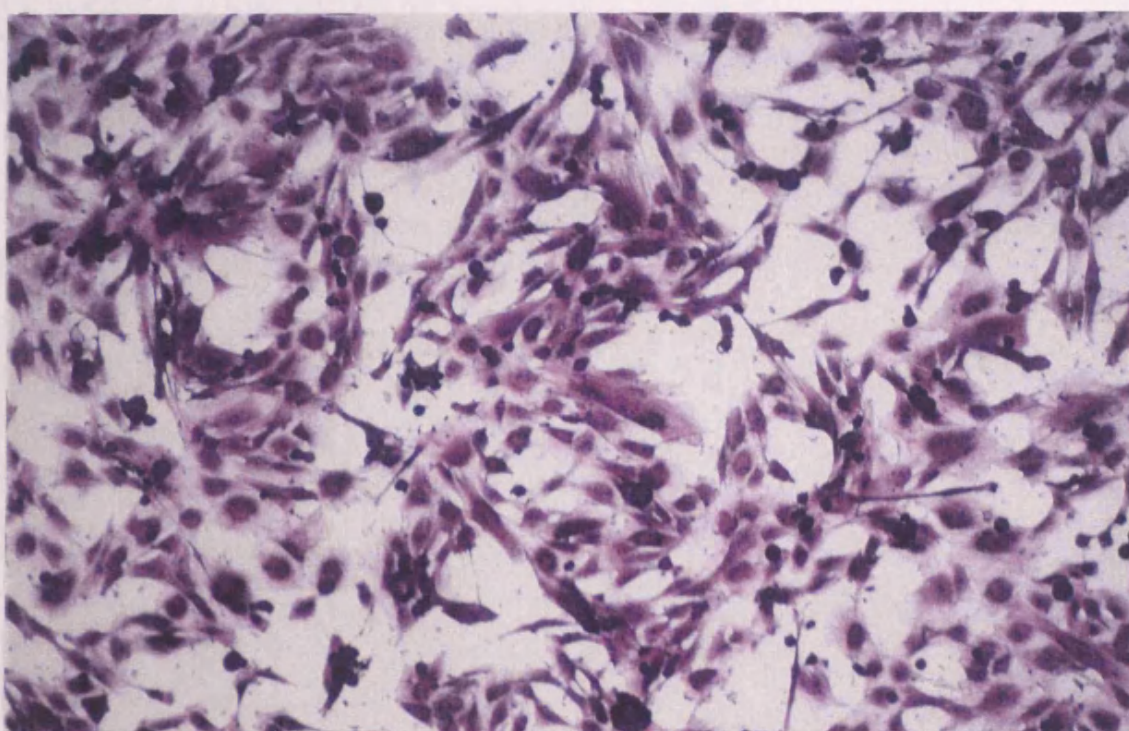


**Figure 5.3** Cytotoxic effect of partially pure preparation of rDNT on EBL cells. A: Confluent monolayer of EBL cells; B: EBL cells treated with partially pure rDNT (magnification x 200).
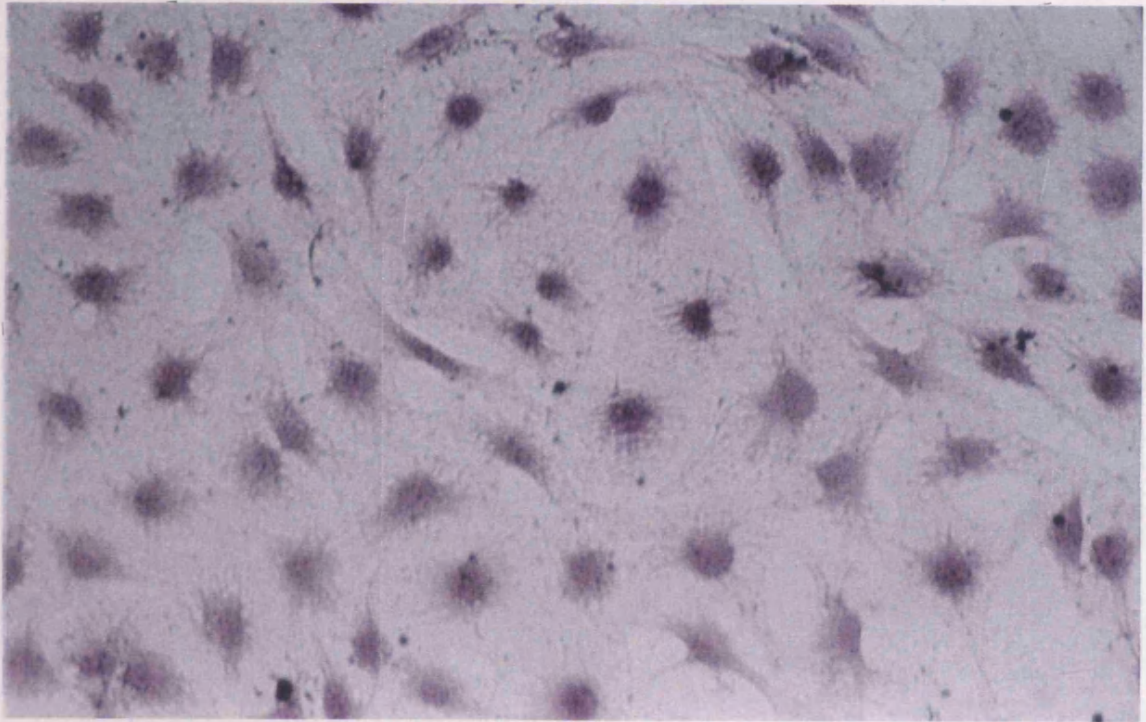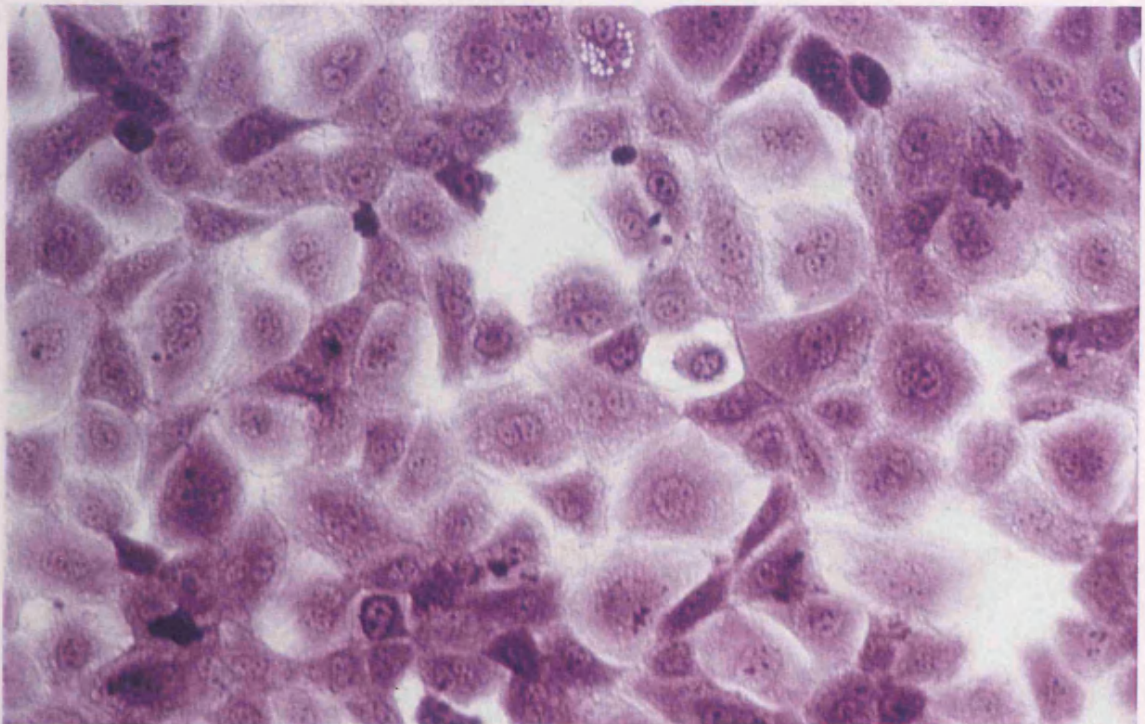
**Figure 5.4** Binucleation effect of partially pure preparations of rDNT on Swiss 3T3 cells. A: Confluent monolayer of Swiss 3T3 cells; B: Swiss 3T3 cells treated with partially pure rDNT; many of the cells are enlarged and binucleated (magnification x 200).

**Figure 5.5** Appearence of binucleated cells in Swiss 3T3 cells under serum-starved conditions with various concentrations of rDNT. The number of cells counted in each field of view ranged from 350 to 550. Each point shows the mean +/- standard deviation of four determinations.

Multinucleation of rDNT treated Swiss 3T3 cells was not observed.

HeLa cells did not show any morphological changes after treatment with rDNT, even at toxin concentrations which would induce morphological changes in EBL cells and Swiss 3T3 cells (data not shown).

## 5.2.2 Effect of rDNT on the cytoskeleton

In order to elucidate the underlying mechanism of rDNT-induced morphological changes, actin stress fibres were stained with rhodamine-phalloidin, and focal adhesions were stained with anti-vinculin and FITC-labelled anti-mouse IgG.

### 5.2.2.1 Effect of rDNT on actin stress fibres

The treatment of sub-confluent, serum-starved Swiss 3T3 cells with rDNT for 24 hours induced the formation of dense thick actin stress fibres, where formation was dose- and time-dependent (Figures 5.6 and 5.7). Control serum-starved Swiss 3T3 cells, not treated with rDNT, had a few thin actin fibres. Sub-confluent Swiss 3T3 cells were treated with various concentrations of rDNT for 24 hours in serum-starved conditions, then the cells were fixed and stained with rhodamine-phalloidin. At 10 ng ml$^{-1}$, the highest concentration of rDNT used, rDNT stimulated the formation of thick, dense actin stress fibres (Figure 5.6). The formation of these stress fibres decreased as the concentration of rDNT decreased. Cells treated with 0.01 ng ml$^{-1}$ rDNT had a few actin fibres and looked like the control cells (Figure 5.6).

At various time points after the addition of 1 ng ml$^{-1}$ rDNT to sub-confluent Swiss 3T3 cells, under serum starved conditions, the cells were fixed and stained. Two and four hours after rDNT treatment the cells looked similar to control cells with a few thin actin fibres (Figure 5.7). After six hours actin stress fibres began to form. Ten hours after treatment with rDNT the cells had dense thick actin stress fibres and looked like cells treated with toxin for 24 hours (Figure 5.7).

10 ng ml$^{-1}$                                0.95 ng ml$^{-1}$
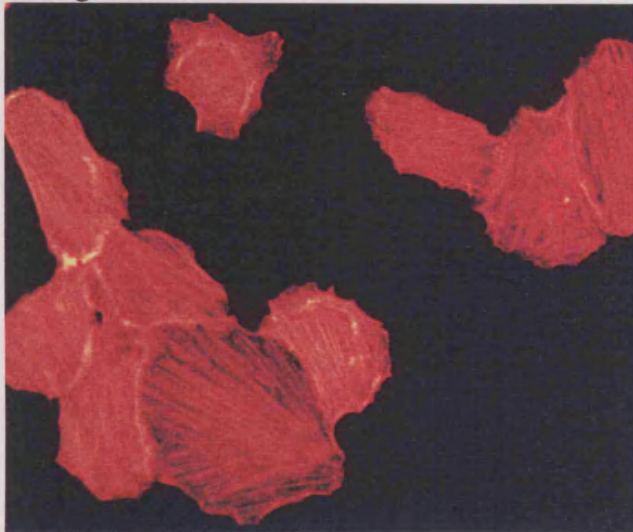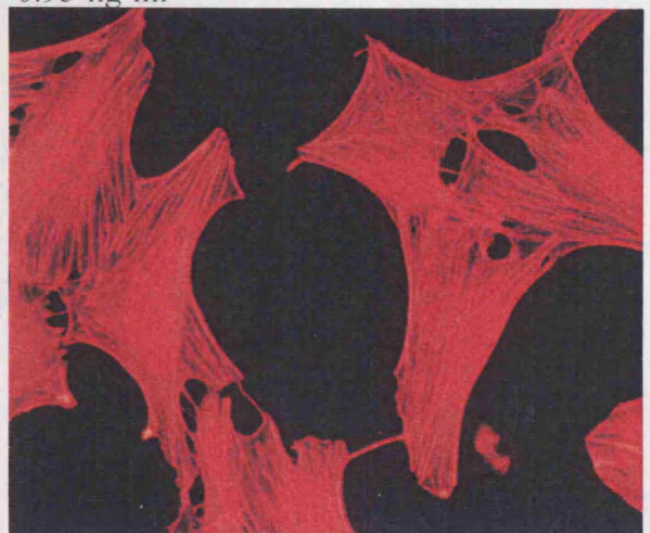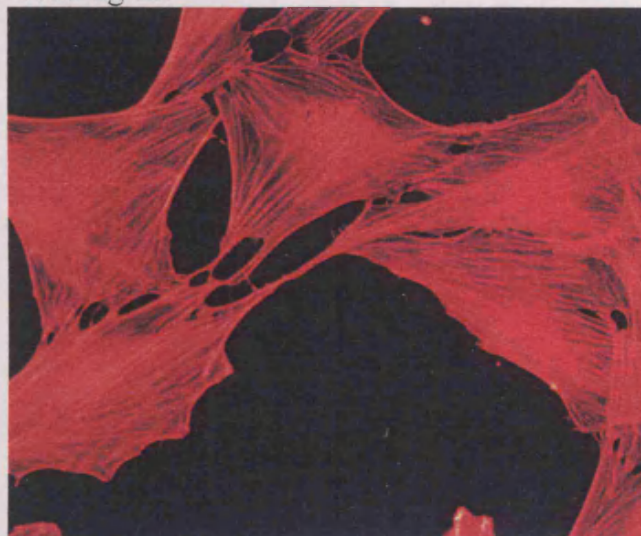
0.16 ng ml$^{-1}$                            0.04 ng ml$^{-1}$
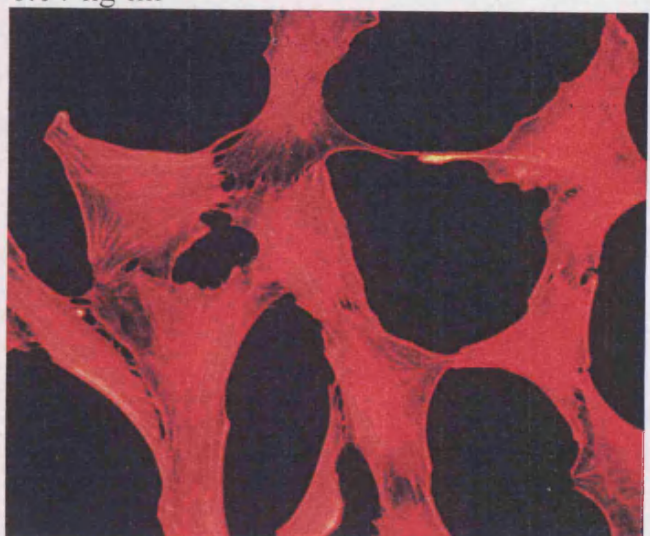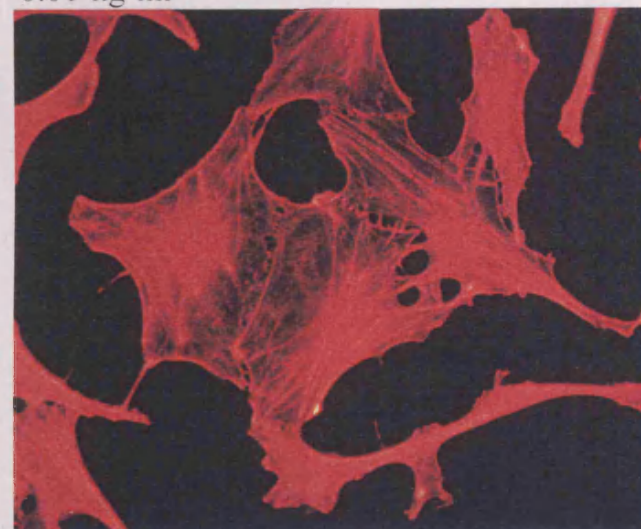
0.01 ng ml$^{-1}$                            0 ng ml$^{-1}$
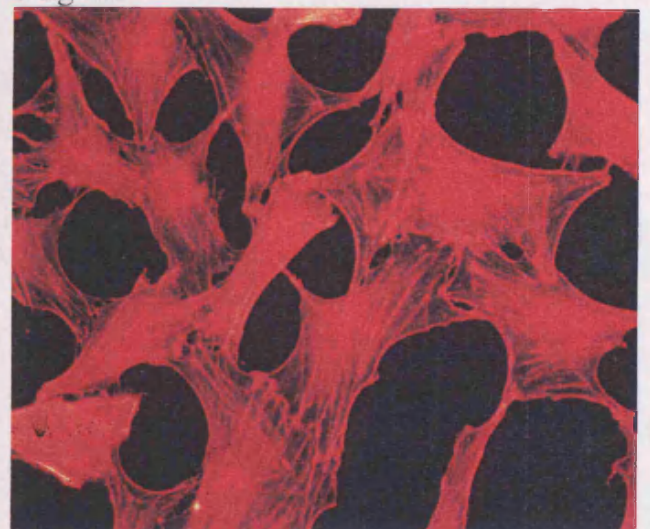


**Figure 5.6** The effect of rDNT on the formation of actin stress fibres in Swiss 3T3 cells. Swiss 3T3 cells were incubated with various concentrations of partially pure rDNT (as indicated on the figure) for 24 hours in serum-starved conditions. After 24 hours the cells were fixed and stained for actin stress fibres with rhodamine-phalloidin (magnification x 400)

2 h
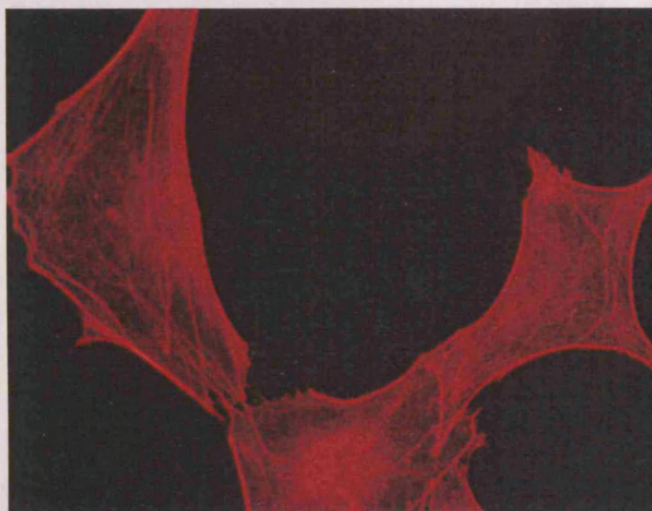
4 h
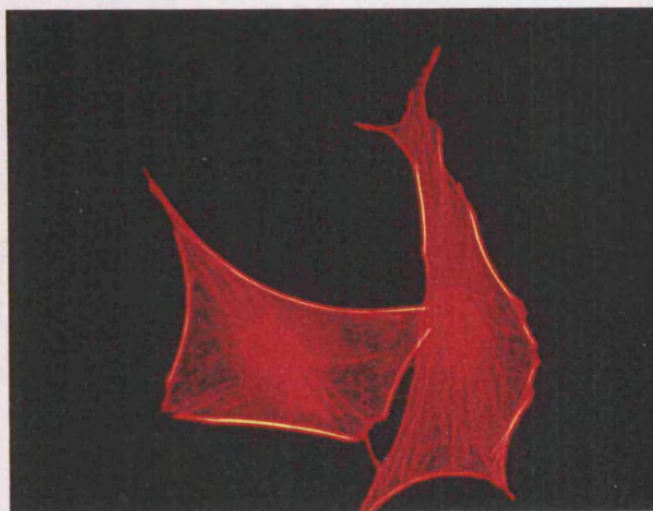
6 h
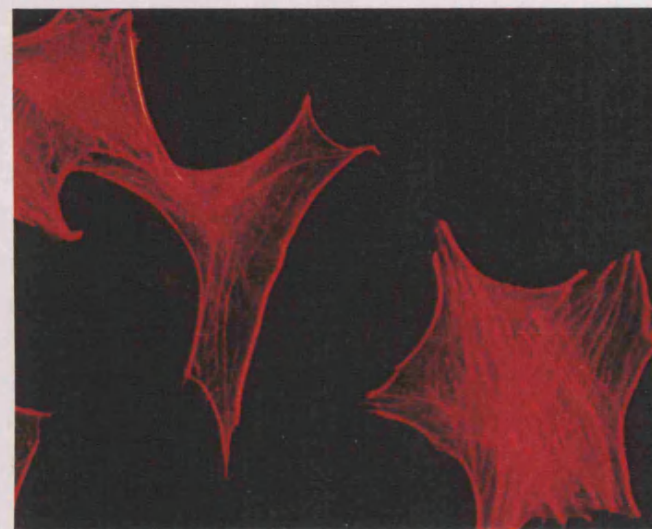
8 h
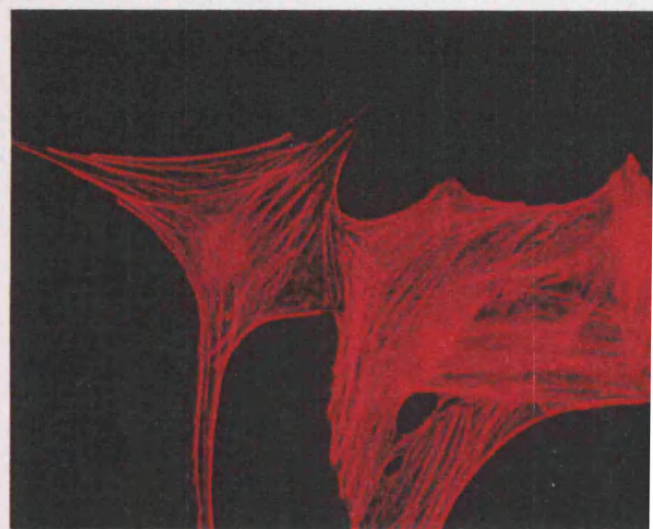
10 h

24 h
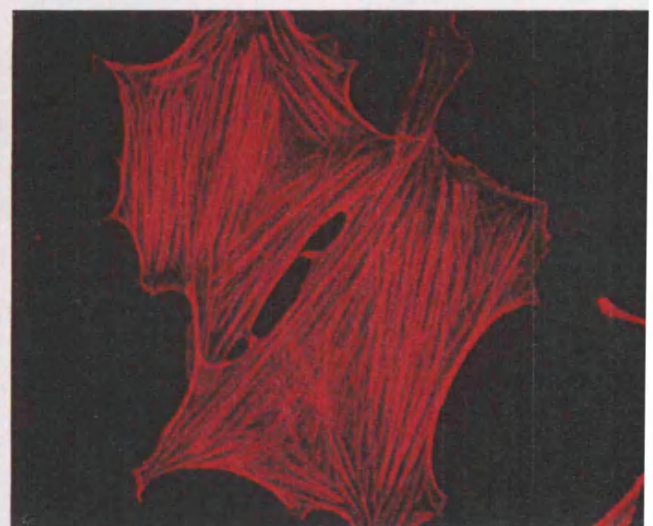
**Figure 5.7** The effect of time on the formation of actin stress fibres induced by rDNT in Swiss 3T3 cells. Swiss 3T3 cells were incubated with 1 ng $ml^{-1}$ rDNT in serum-starved conditions. At various time points (as indicated in the figure) after the addition of rDNT the cells were fixed and stained with rhodamine-phalloidin for actin stress fibres (magnification x 400).

## 5.2.2.2 Effect of rDNT on focal adhesions

Initial attempts to stain Swiss 3T3 cells by immunofluorescence for the presence of vinculin-containing focal adhesions were unsuccessful. The FITC-labelled goat anti-mouse IgG antibody (Sigma), which is used to bind to the mouse monoclonal anti-vinculin (clone VIN-11-5, Sigma), bound non-specifically to the murine Swiss 3T3 cells. This caused high background fluorescence even after the cells were blocked with 5% goat serum. This nonspecific binding was prevented by blocking the cells by preincubation with 5% donkey serum and using a FITC-labelled donkey anti-mouse IgG antibody (Jackson Immuno Research Lab. Inc.) to bind to the anti-vinculin monoclonal.

Recombinant DNT stimulated the formation of focal adhesions in sub-confluent Swiss 3T3 cells under serum-starved conditions after 24 hours. Control cells, not treated with rDNT, had few vinculin-containing focal adhesions. The formation of these focal adhesions was dose and time dependant (Figures 5.8 and 5.9). Cells treated with 10 ng ml$^{-1}$ rDNT showed the formation of many focal adhesions (Figure 5.8). The formation of focal adhesions decreased as the concentration of rDNT decreased. At 0.04 ng ml$^{-1}$ rDNT the toxin-treated cells looked like the control cells (Figure 5.8). The concentration of rDNT required to stimulate the formation of focal adhesions was similar to the concentration of rDNT required for the formation of actin stress fibres.

Sub-confluent Swiss 3T3 cells under serum starved conditions were treated with 1 ng ml$^{-1}$ rDNT, and at various intervals after the addition of rDNT the cells were fixed and stained. After two, four and six hours of rDNT treatment the cells had a few focal adhesions and looked similar to control cells (Figure 5.9). After eight hours the number of focal adhesions increased, reaching a maximum at ten hours when the toxin treated cells had many focal adhesions and look similar to cells treated with rDNT for 24 hours (Figure 5.9).

Double staining of Swiss 3T3 cells with rhodamine-phalloidin, and anti-vinculin and FITC-labelled anti-mouse IgG, showed that rDNT-treated cells had many focal adhesions which were observed at the end of thick actin stress fibres (Figure 5.10). In contrast, untreated control cells had a few focal adhesions which were found at the ends of rare, thin actin stress fibres (Figure 5.10).

10 ng ml$^{-1}$
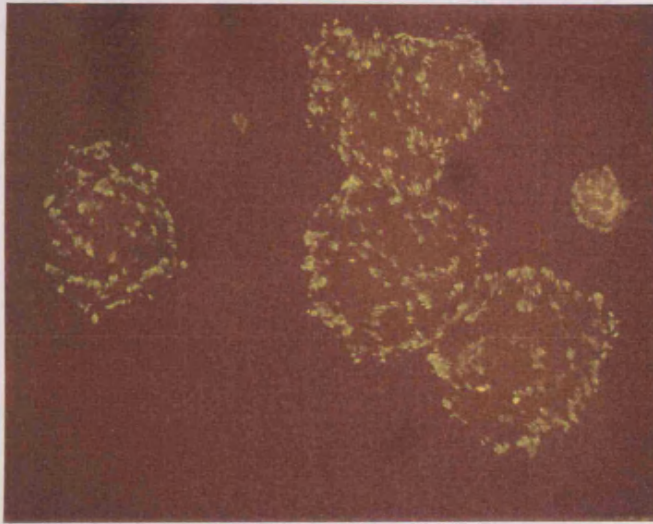
0.95 ng ml$^{-1}$
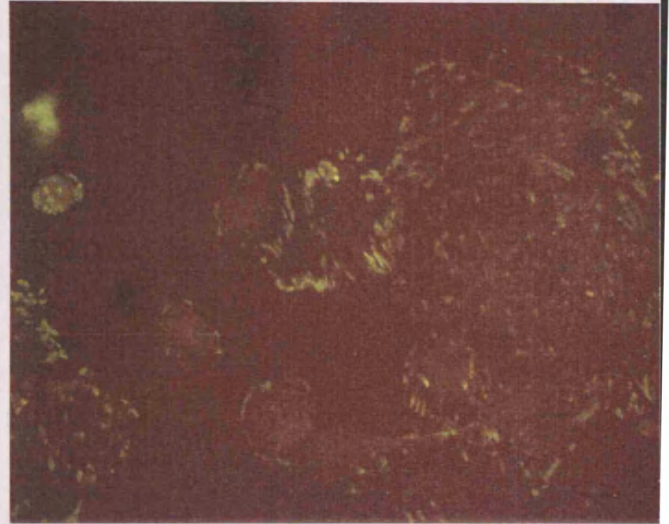
0.16 ng ml$^{-1}$
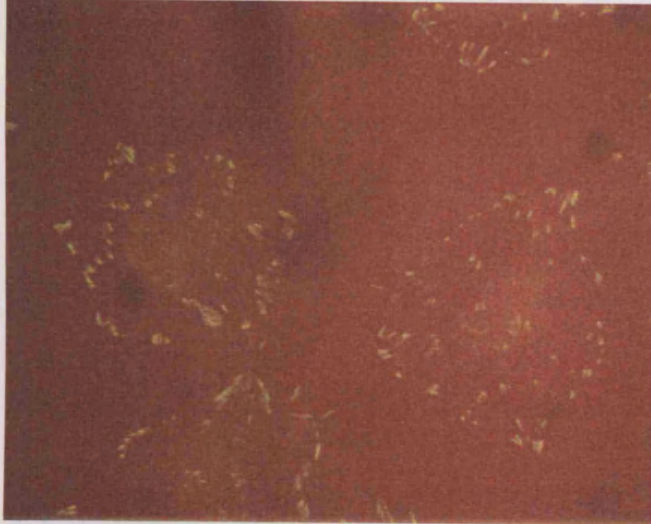
0.04 ng ml$^{-1}$

0.01 ng ml$^{-1}$
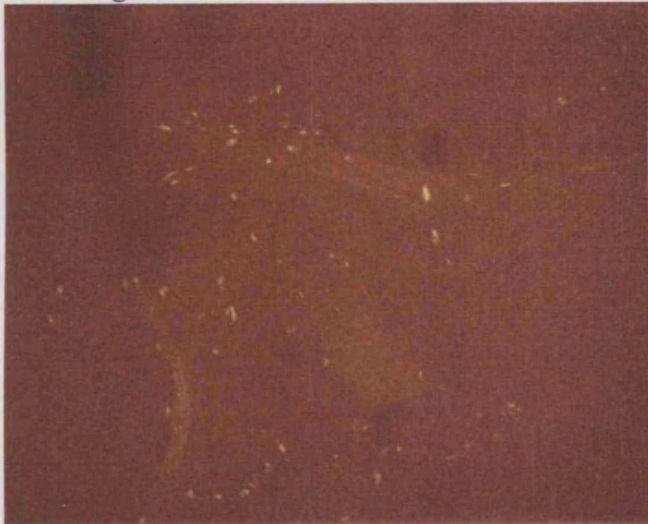
0 ng ml$^{-1}$
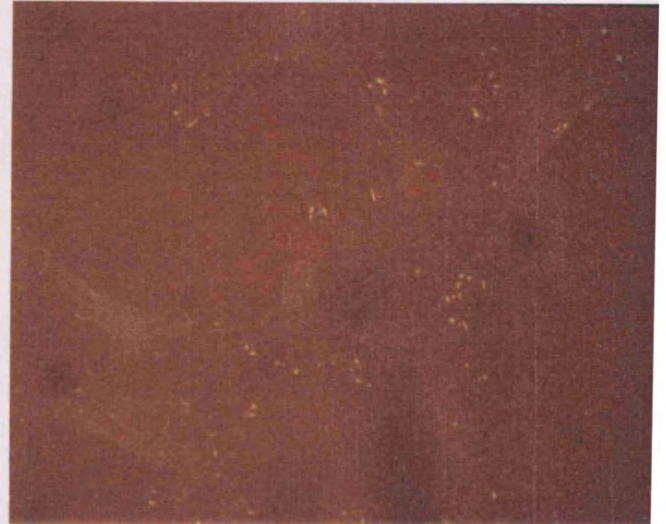
**Figure 5.8** The effect of rDNT on the formation of focal adhesions in Swiss 3T3 cells. Swiss 3T3 cells were incubated with various concentrations of partially pure rDNT (as indicated on the figure) for 24 hours in serum-starved conditions. After 24 hours the cells were fixed and stained for focal adhesions with anti-vinculin monoclonal and FITC-labelled anti-mouse IgG antibody (magnification x 400).
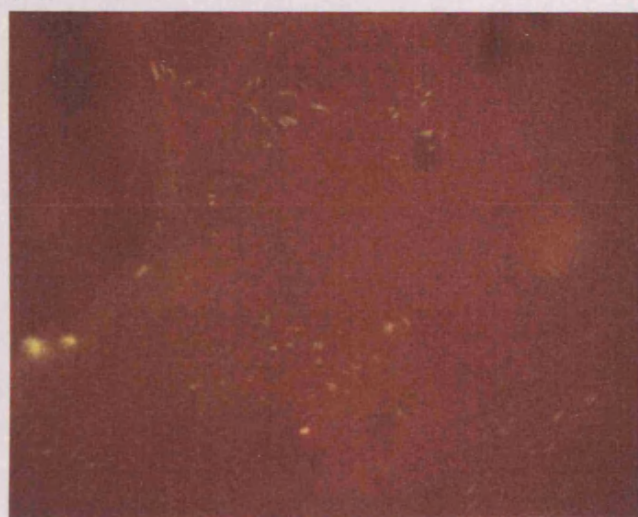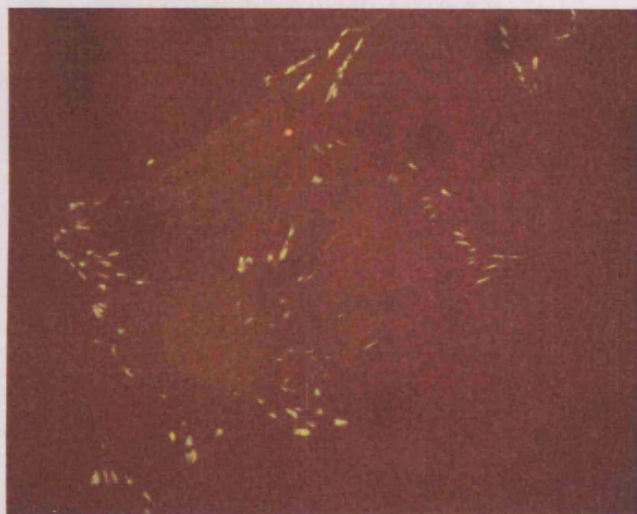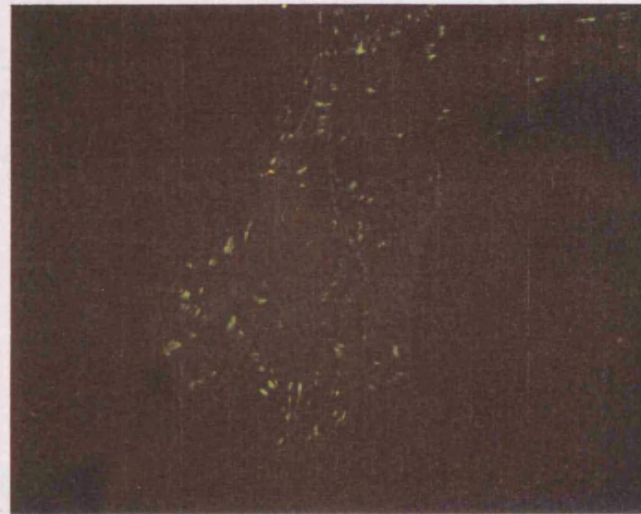
**Figure 5.9** The effect of time on the formation of focal adhesions induced by rDNT in Swiss 3T3 cells. Swiss 3T3 cells were incubated with 1 ng ml$^{-1}$ rDNT in serum-starved conditions. At various time points (as indicated in the figure) after the addition of rDNT the cells were fixed and stained with focal adhesions with anti-vinculin monoclonal and FITC-labelled anti-mouse IgG antibody (magnification x 400).
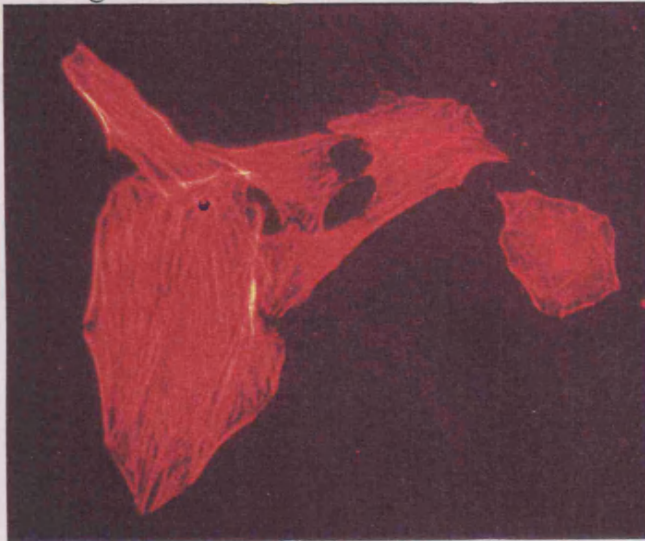
A 1 ng ml$^{-1}$ rDNT

A 0 ng ml$^{-1}$ rDNT
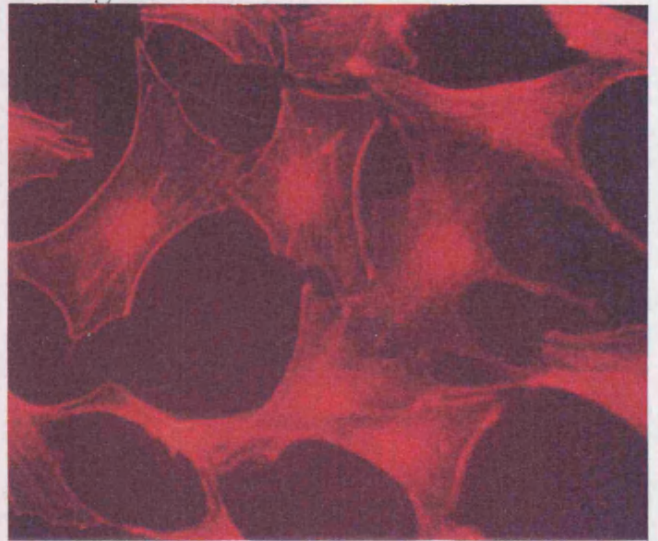
B 1 ng ml$^{-1}$ rDNT
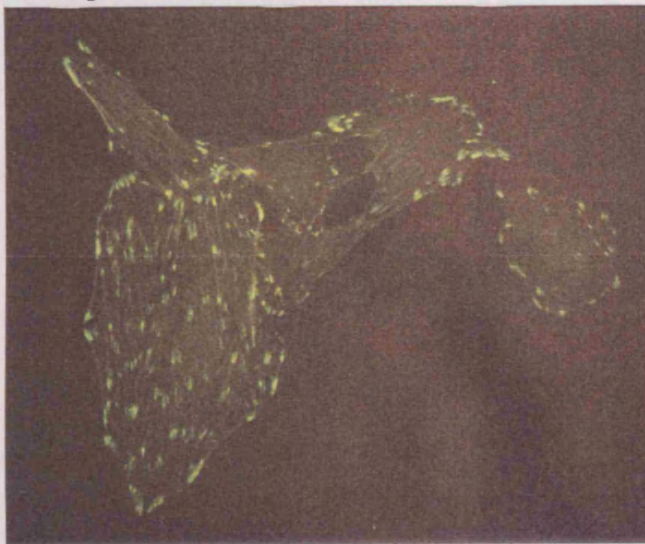
B 0 ng ml$^{-1}$ rDNT
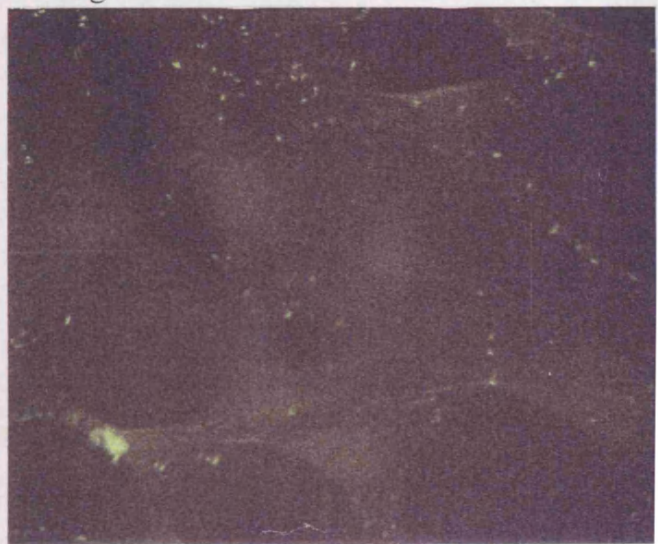
**Figure 5.10** Double staining of rDNT-treated and control Swiss 3T3 cells for actin stress fibres and focal adhesions. Swiss 3T3 cells were incubated with or without 1 ng ml$^{-1}$ rDNT in serum-starved condition. After 24 hours the cells were fixed and stained with rhodamine-phalloidin, and anti-vinculin monoclonal and FITC-labelled anti-mouse IgG antibody. A: cells stained for actin stress fibres; B: the same cells stained for focal adhesions (magnification x 400).

### 5.2.2.3 Effects of purified XL1-blue/bluescript and K1310A on actin stress fibres and focal adhesions

Sub-confluent, serum-starved Swiss 3T3 cells were treated with various concentrations of purified proteins from XL1-blue/bluescript and purified mutant rDNT from K1310A for 24 hours, then stained with rhodamine-phalloidin or anti-vinculin and FITC-labelled anti-mouse IgG. The total protein concentration of the samples was determined, 510 $\mu$g ml$^{-1}$ and 250 $\mu$g ml$^{-1}$ respectively. The concentration of mutant rDNT from K1310A was estimated as described for rDNT in Section 4.2.6. XL1-blue/bluescript contained no rDNT visible by SDS-PAGE, therefore 10% of the total protein concentration was used as an approximate.

Purified XL1-blue/bluescript proteins and mutant rDNT from K1310A did not stimulate the formation of actin stress fibres or focal adhesions in Swiss 3T3 cells (Figures 5.11 and 5.12). Even at approximate rDNT concentrations as high as 1.25 $\mu$g ml$^{-1}$ for mutant rDNT from K1310A and 3.1 $\mu$g ml$^{-1}$ for XL1-blue/bluescript no effect was seen. These protein concentration were about 1,000 times and 3,000 times higher, respectively, than the concentration of protein required to stimulate actin stress fibre and focal adhesion formation by rDNT. Cells treated with XL1-blue/bluescript or K1310A had few focal adhesions and some thin actin fibres, and looked similar to the control cells.

### 5.2.3 Stimulation of DNA synthesis in Swiss 3T3 cells by rDNT

### 5.2.3.1 The ability of sonicated cell extracts from DNT1, B58 and XL1-blue/bluescript to stimulate DNA synthesis in Swiss 3T3 cells

This work was performed in collaboration with Theresa Higgins at ICRF, London. In a preliminary study sonicated cell extracts from strains DNT1, *B. bronchiseptica* B58 and XL1-blue/bluescript were assayed on quiescent Swiss 3T3 cells for their ability to stimulate DNA synthesis. The total protein concentrations of the samples were 740 $\mu$g ml$^{-1}$, 370 $\mu$g ml$^{-1}$, 780 $\mu$g ml$^{-1}$ respectively. Samples were diluted in DMEM to give the same protein concentrations, and each sample was assayed in duplicate. Replicate experiments gave similar curves and Figure 5.13 shows the results of a representative experiment. XL1-blue/bluescript sonicated cell extract did not stimulate DNA synthesis. The sonicated cell extracts of strains B58 and DNT1

A
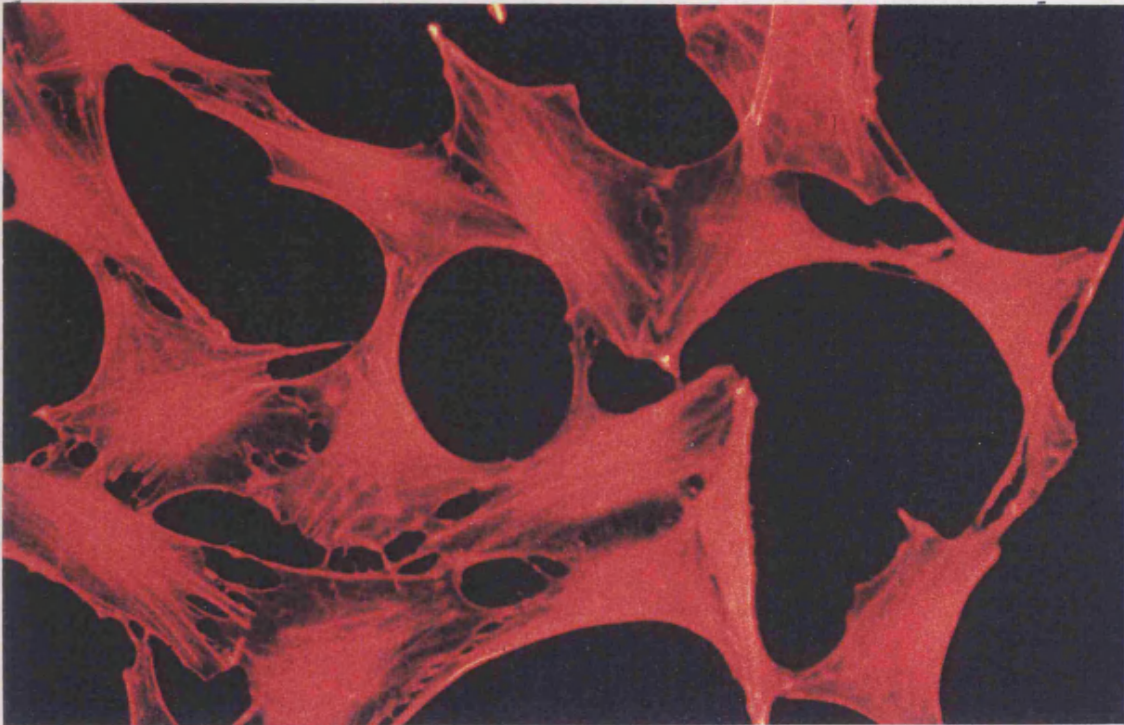


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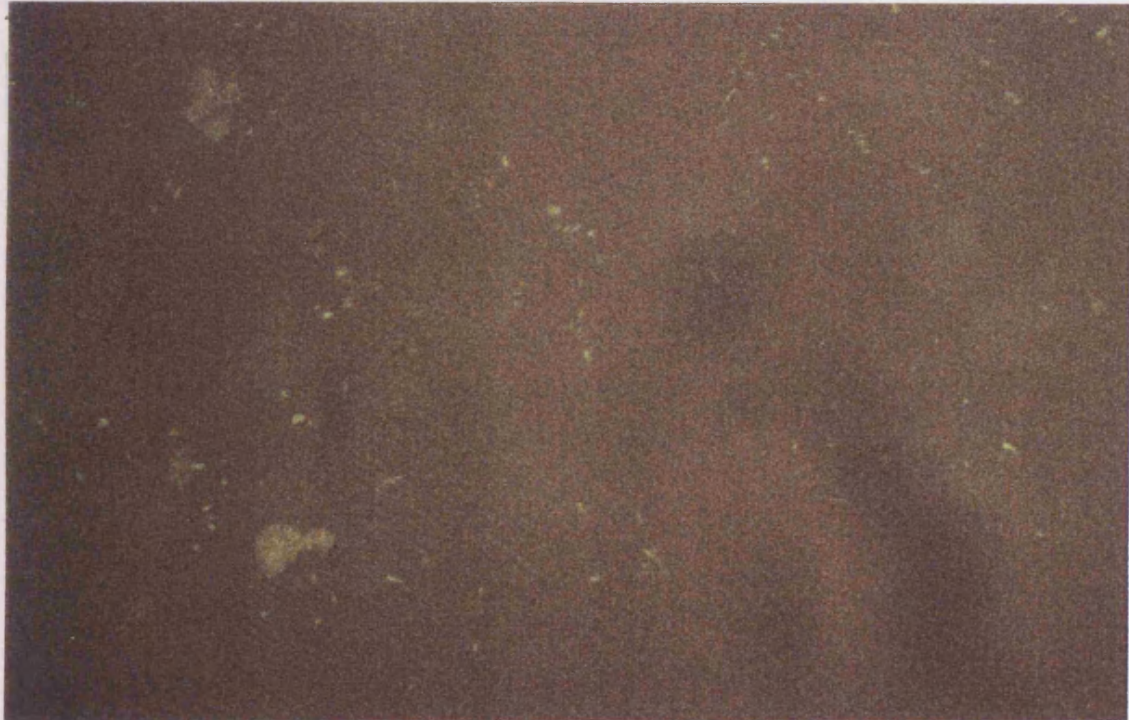


**Figure 5.11** The effect of partially pure preparations of XL1blue/bluescript on the formation of actin stress fibres and focal adhesions in Swiss 3T3 cell. Swiss 3T3 cells were incubated with 3.1 μg ml$^{-1}$ XL1blue/bluescript in serum-starved conditions for 24 hours. A: the cells were fix and stained with rhodamine-phalloidin for actin stress fibres; B: the cells were fixed and stained with anti-vincul in monoclonal and FITC-labelled anti-mouse IgG antibody (magnification x 400).
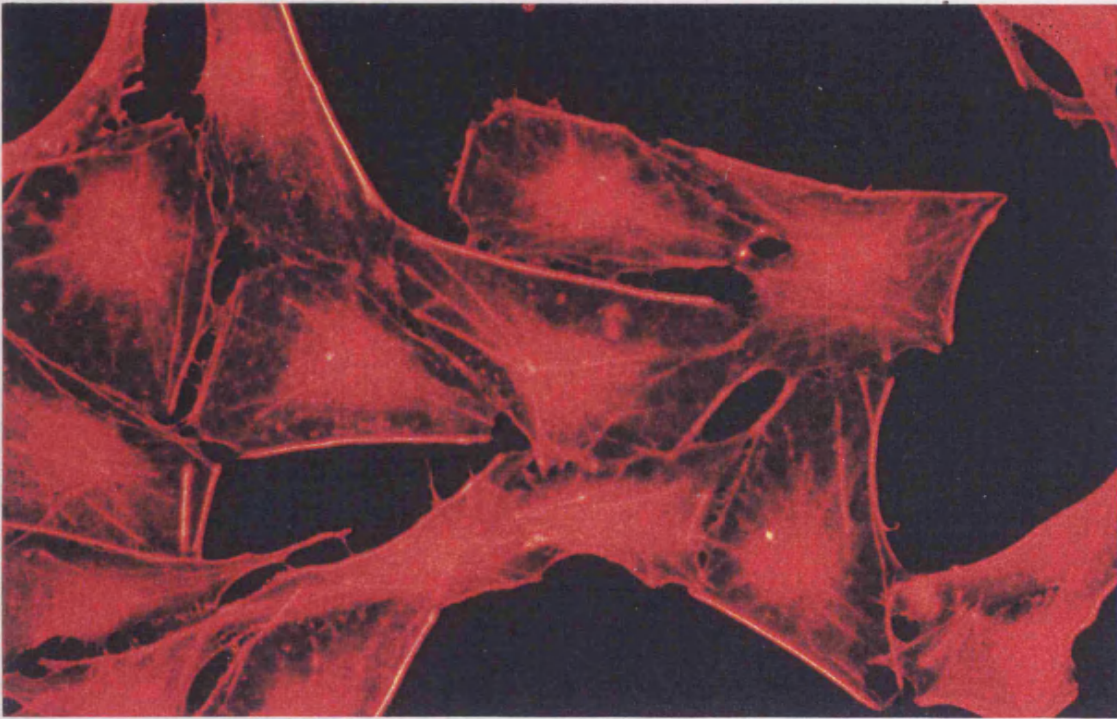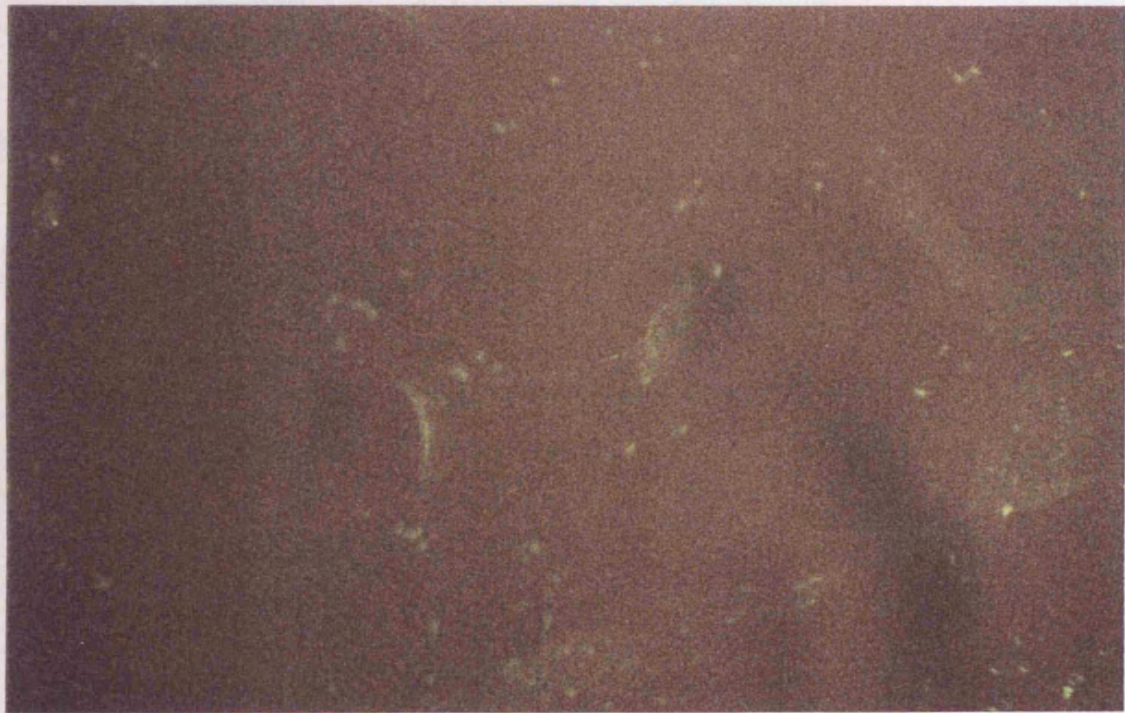
**Figure 5.12** The effect of partially pure preparations of mutant rDNT from strain K1310A on the formation of actin stress fibres and focal adhesions in Swiss 3T3 cell. Swiss 3T3 cells were incubated with 1.25 µg ml⁻¹ mutant rDNT from K1310A in serum-starved conditions for 24 hours. A: the cells were fix and stained with rhodamine-phalloidin for actin stress fibres; B: the cells were fixed and stained with anti-vinculin monoclonal and FITC-labelled anti-mouse IgG antibody (magnification x 400).
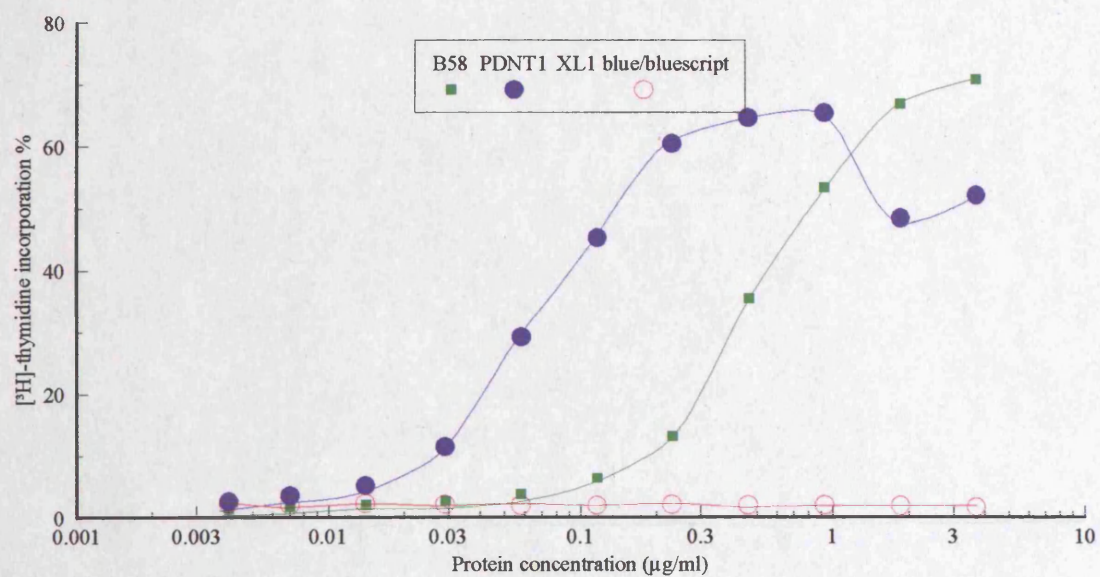
**Figure 5.13** Dose response curve showing stimulation of DNA synthesis in quiescent Swiss 3T3 cells by sonicated cell extracts of *B. bronchiseptica* strains B58, DNT1 and *E. coli* XL1-blue/bluescript. The level of [³H]-thymidine incorporation is expressed as a percentage of the incorporation induced by 10% FCS (62,348 cpm). Each point is the mean of two determinations.

induced [³H]-thymidine incorporation into quiescent Swiss 3T3 cells, indicating that DNA synthesis was stimulated by wild-type DNT and rDNT. Strain B58 stimulated DNA synthesis to 70% of that obtained with 10% FCS, while the maximal stimulation by strain DNT1 was 65% of that obtained with 10% FCS. The level of stimulation fell slightly at higher concentrations, possibly due to the toxicity of DNT to Swiss 3T3 cells.

The maximum level of DNA synthesis stimulated by 10% FCS or rDNT samples varied between separate experiments. Despite this variation the trends in each experiment were similar, i.e. DNA synthesis increased as the concentration of rDNT increased. Factors which could vary between experiments, and therefore affect results, included the passage number of Swiss 3T3 cells and the stock FCS serum used to induce maximal stimulation.

## 5.2.3.2 The ability of partially purified rDNT to stimulate DNA synthesis in Swiss 3T3 cells

A dose response curve for the stimulation of DNA synthesis by partially pure rDNT was constructed. The concentration of rDNT was estimated as described in Section 4.2.6. The sample was diluted in DMEM and added to the Swiss 3T3 cells to give an estimated final concentration range of 0.0025-40 ng ml$^{-1}$, and all samples were tested in triplicate. Figure 5.14 shows the stimulation of DNA synthesis by rDNT at various concentrations from three different experiments. Optimal stimulation was obtained at a rDNT concentration of 40 ng ml$^{-1}$ and was equivalent to approximately 80% of the stimulation of DNA synthesis obtained with 10% FCS. A half maximal effect was obtained at a concentration of approximately 1 ng ml$^{-1}$.

## 5.2.3.3 Heat lability of the DNA synthesis stimulating activity of rDNT in Swiss 3T3 cells

DNT is known to be a heat-labile toxin (Munoz, 1971), and experiments were performed to see if the induction of DNA synthesis by rDNT was heat-labile. Partially purified rDNT was heated at 56°C for 30 minutes and diluted in DMEM to give an estimated final concentration range of 0.0025-40 ng ml$^{-1}$. Heat-treated rDNT was unable to stimulate DNA synthesis in Swiss 3T3 cells (Figure 5.15), even at 40 ng
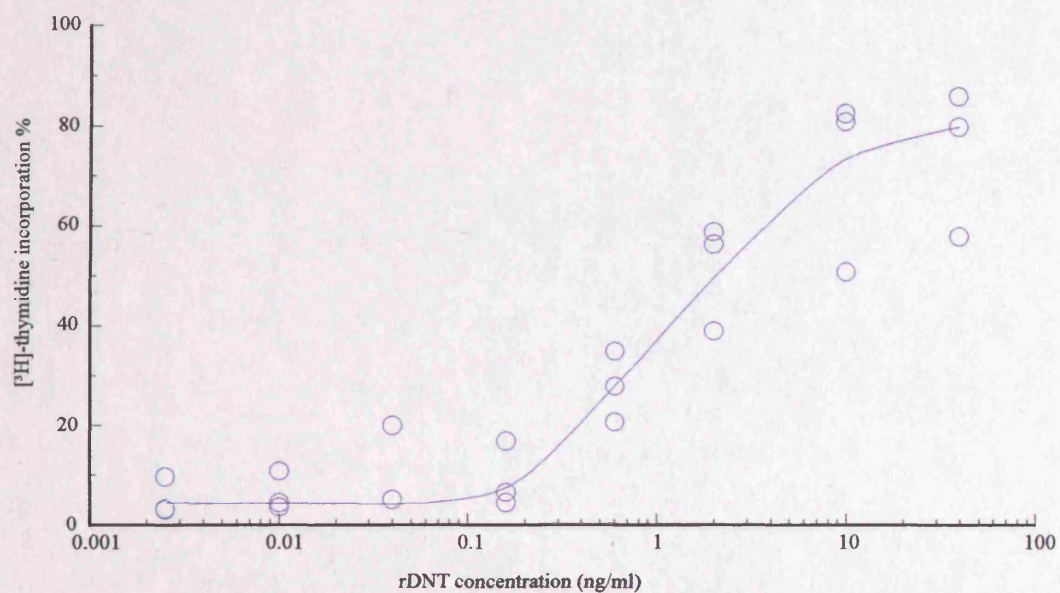
**Figure 5.14** Dose response curve showing stimulation of DNA synthesis in quiescent Swiss 3T3 cells by partially pure rDNT. The level of [³H]-thymidine incorporation is expressed as a percentage of the incorporation induced by 10% FCS. Each concentration of rDNT was tested in three different experiments, and each point is the mean of three determinations.
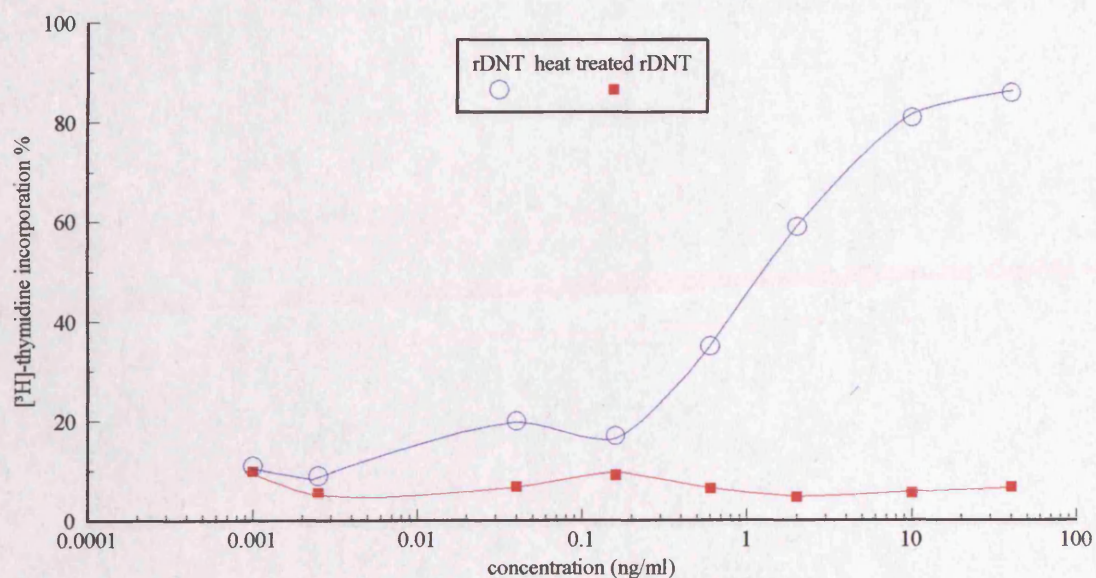


**Figure 5.15** Effect of heating partially pure rDNT on stimulation of DNA synthesis in quiescent Swiss 3T3 cells. Partially pure rDNT was heated to 56°C for 30 minutes, then various concentrations were tested on Swiss 3T3 cells. The level of [³H]-thymidine incorporation is expressed as a percentage of the incorporation induced by 10% FCS (50,000 cpm). Each point is the mean of three determinations.

ml$^{-1}$ the concentration of unheated rDNT that stimulated maximal DNA synthesis.

## 5.2.3.4 The ability of partially purified XL1-blue/bluescript and K1310A to stimulate DNA synthesis in Swiss 3T3 cells

The ability of partially purified XL1-blue/bluescript proteins and mutant rDNT from strain K1310A to stimulate DNA synthesis in Swiss 3T3 cells was investigated. The samples were diluted in DMEM to give a final estimated concentration range between 0.003-30 ng ml$^{-1}$. Figure 5.16 shows that purified proteins from XL1-blue/bluescript and mutant rDNT from strain K1310A did not stimulate DNA synthesis in quiescent Swiss 3T3 cells, even at protein concentrations of 40 ng ml$^{-1}$.

## 5.2.3.5 Stimulation of DNA synthesis by transient exposure to rDNT

To test whether the continuous presence of rDNT in the extracelluar medium was essential for the stimulation of DNA synthesis, cells were washed after incubating them with rDNT for various periods. Then, cells were incubated in serum-starved conditions up to 40 hours and the rate of [$^3$H]-thymidine incorporation was determined (Figure 5.17). DNA synthesis was stimulated in cells exposed to rDNT for 1 hour, the percentage DNA synthesis was 67% of that obtained in cells exposed to rDNT for 40 hours. Maximum DNA synthesis occurred after 3 hours exposure to rDNT, with a slight decrease between 3 and 40 hours.

## 5.2.3.6 Effect of methylamine on the stimulation of DNA synthesis by rDNT

The entry of many bacterial toxins into the cytoplasm is prevented by the addition of membrane-permeant weak bases such as methylamine. I first investigated the concentrations of methylamine that could inhibit the stimulation of DNA synthesis by 10 ng ml$^{-1}$ rDNT. It was known that 9 mM methylamine inhibited the stimulation of DNA synthesis by PMT (Rozengurt *et al.*, 1990), and so this was used as a control to ensure the assay was working. Figure 5.18 shows that methylamine inhibited stimulation of DNA synthesis by rDNT in a concentration dependent manner, except at the highest methylamine concentrations when DNA synthesis was not inhibited. This was despite the fact that 9 mM methylamine did inhibit the stimulation of DNA synthesis by 10 ng ml$^{-1}$ PMT (Figure 5.18). DNA synthesis was inhibited four-fold by
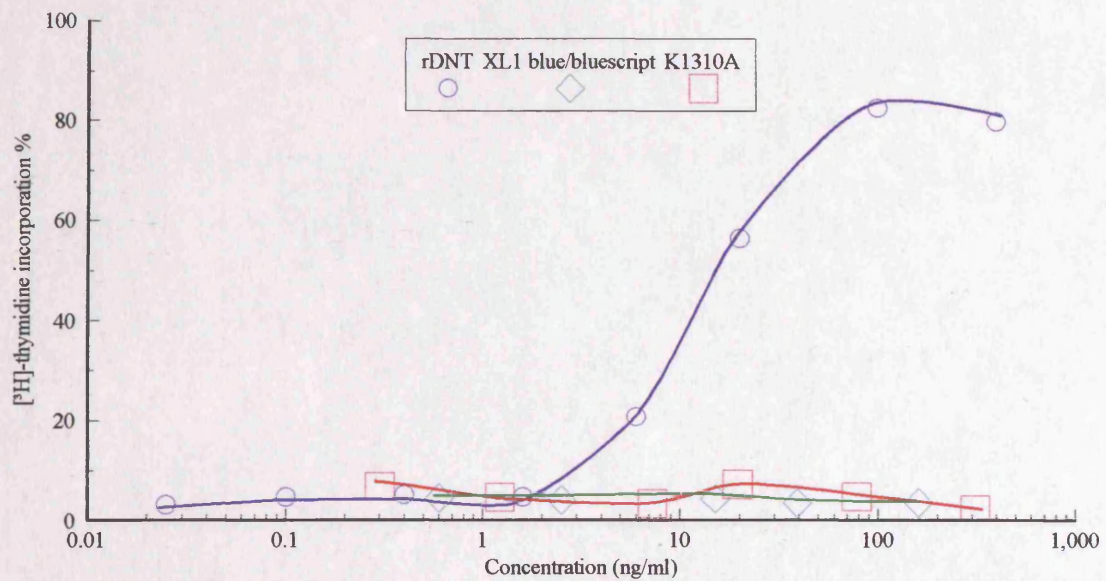
**Figure 5.16** The ability of partially purified proteins from strains XL1-blue/bluescript and K1310A to stimulate DNA synthesis in quiescent Swiss 3T3 cells. The level of [$^3$H]-thymidine incorporation is expressed as a percentage of the incorporation induced by 10% FCS (55,000 cpm). Each point is the mean of three determinations.
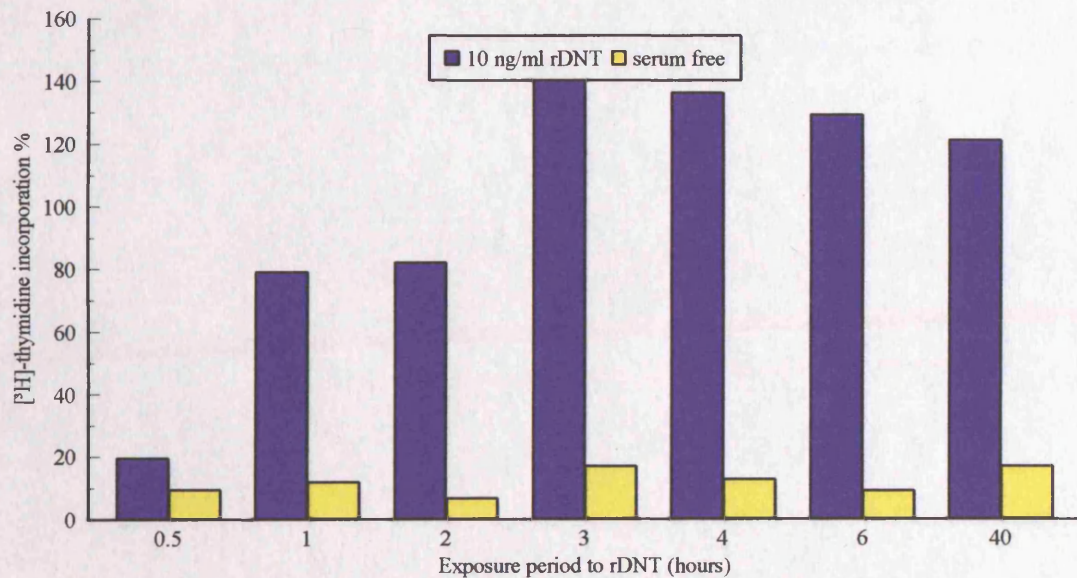


**Figure 5.17** Effect on DNA synthesis in quiesent Swiss 3T3 cells to transient exposure of 10 ng/ml rDNT. The level of [$^3$H]-thymidine incorporation is expressed as a percentage of the incorporation induced by 10% FCS (29,000 cpm). Each point is the mean of three determinations.

7 mM methylamine. Replicated experiments gave similar curves and Figure 5.18 represents a typical experiment.

To investigate if methylamine had a direct effect on DNA synthesis, various concentrations of methylamine without rDNT were assayed on Swiss 3T3 cells. It was found that the addition of 2 to 9 mM methylamine to quiescent Swiss 3T3 cells stimulated DNA synthesis to approximately 20% of that obtained with 10% FCS, about four times higher than that obtained for serum-starved cells without methylamine. At 10 mM methylamine DNA synthesis was stimulated to 38% of that obtained for 10% FCS, nearly half the DNA synthesis stimulated by rDNT (data not shown). These controls suggest that the methylamine was directly affecting DNA synthesis of the Swiss 3T3 cells. Methylamine is a weak base and if the cell medium becomes basic this may stimulate DNA synthesis in Swiss 3T3 cell (T. Higgins, personal communication). This would also explain why methylamine inhibited the activity of rDNT in a dose-dependent manner except at the highest concentrations of methylamine when the level of DNA synthesis increased.

Figure 5.19 shows that 7 mM methylamine became less inhibitory as the time interval between the addition of rDNT and methylamine increased. Methylamine did not inhibit DNA synthesis when added 2 hours after the addition of rDNT. But DNA synthesis was not completely inhibited even when methylamine and rDNT were added simultaneously.

### 5.2.4 Effect of rDNT on cell proliferation

Sub-confluent Swiss 3T3 cells were incubated in serum-starved conditions with or without 2.5 ng ml$^{-1}$ rDNT, or in the presence of 10% FCS for 6 days. Every 24 hours the cells were counted as described in Section 2.8.10. Figure 5.20 shows the results of a representative experiment, and each point indicates the mean of four determinations. Replicate experiments gave similar curves. The number of cells incubated in serum-starved conditions with or without 2.5 ng ml$^{-1}$ rDNT fell, while the number of cells incubated with 10% FCS increased about sevenfold.
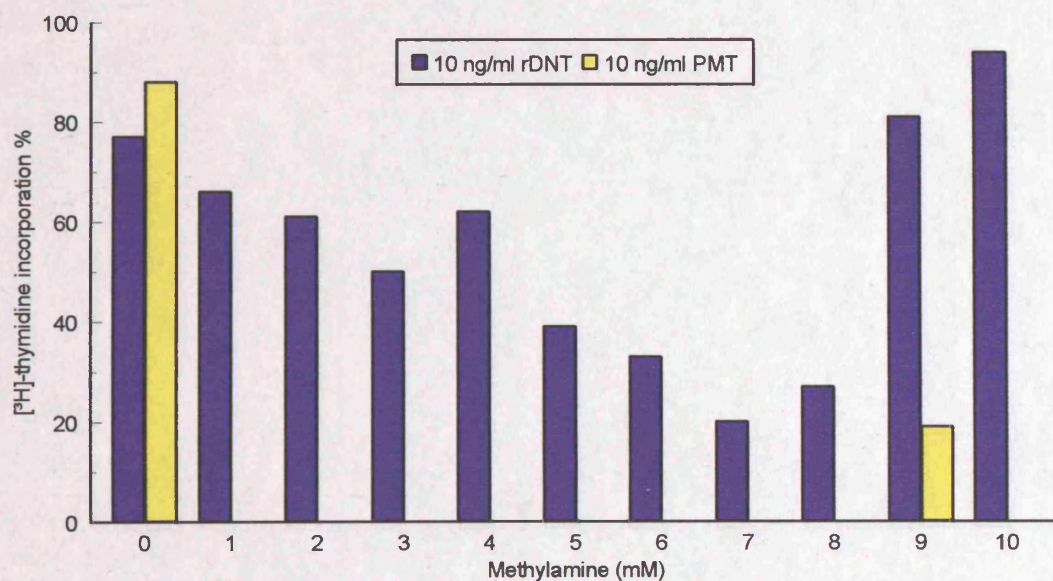
**Figure 5.18** Effect of various concentrations of methylamine on DNA synthesis stimulated by 10 ng/ml rDNT in quiescent Swiss 3T3 cells. The level of [$^3$H]-thymidine incorporation is expressed as a percentage of the incorporation induced by 10% FCS (54,000 cpm). Each point is the mean of three determinations. PMT was purified by P. Ward as described in Ward *et al.* (1994).
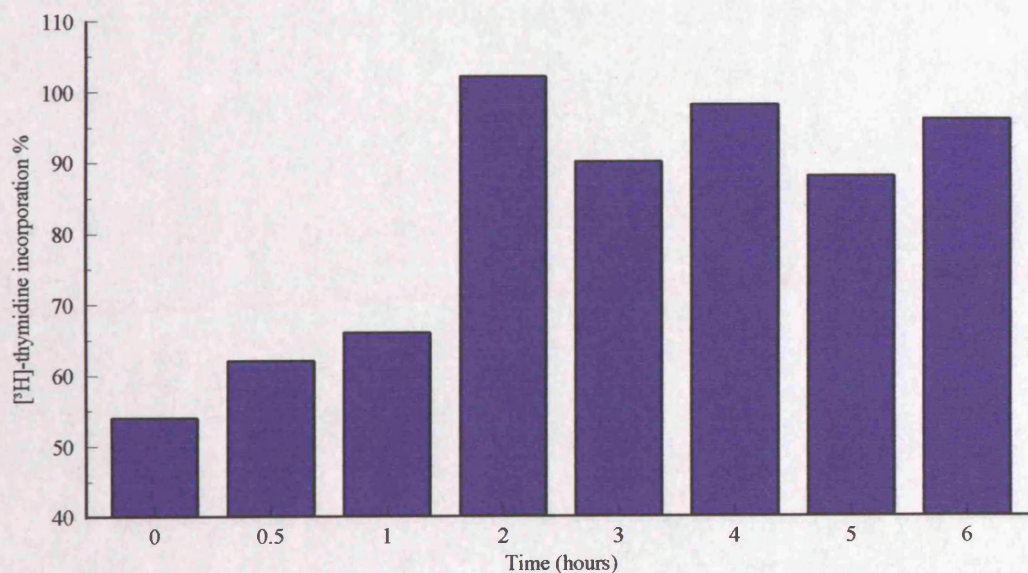


**Figure 5.19** Effect of 7 mM methylamine on the stimulation of DNA synthesis in quiescent Swiss 3T3 cells by 10 ng/ml rDNT added at various times after the addition of rDNT. The level of [$^3$H]-thymidine incorporation is expressed as a percentage of the incorporation induced by 10% FCS (39,000 cpm). Each time point is the mean of three determinations.
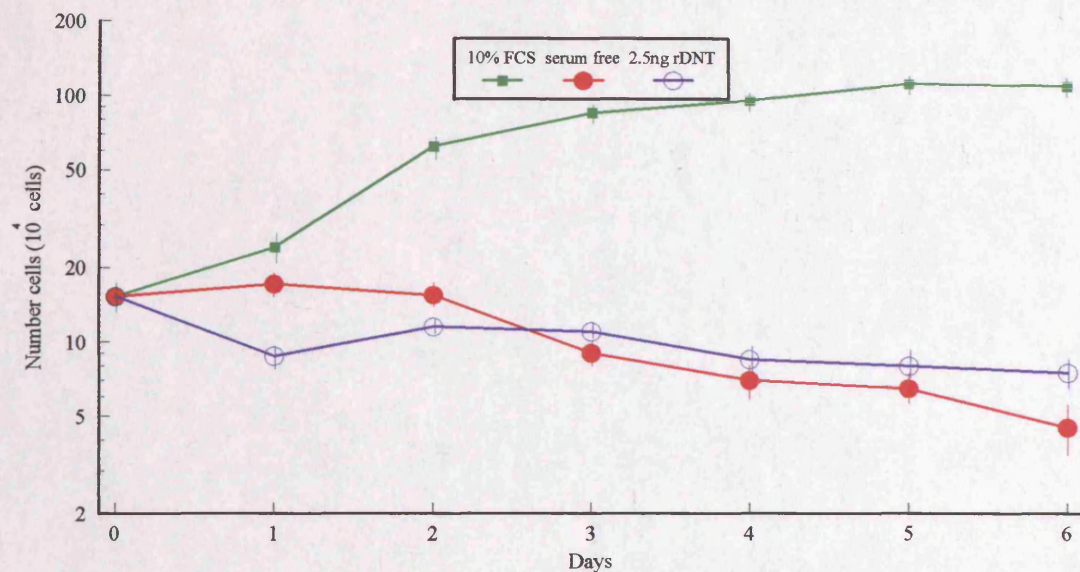
**Figure 5.20** The effect of rDNT on the proliferation of Swiss 3T3 cells. Cells were plated at a density of 30,000 cells per well and incubated for 24 hours. The cells were washed and incubated with DNT or 10% FCS for indicated periods. The cells were counted and each point indicates the mean +/- standard deviation of four determinations.

### 5.2.5 Modification of Rho by rDNT in vivo

In our laboratory crude lysates of DNT1 have been shown to modify recombinant Rho (G. Pullinger, personal communication). In a attempt to show whether rDNT can modify Rho *in vivo*, crude lysates were prepared from Swiss 3T3 cells treated with 10 ng ml$^{-1}$ rDNT and without rDNT in serum starved conditions for 24 hours. The crude lysates were screened by Western blot analysis with an anti-RhoA monoclonal (26C4) (Santa Cruz Biotechnology, USA). Purified recombinant RhoA (from Professor A. Hall, London) was used as a positive control to show that the monoclonal recognised RhoA. Western blot analysis showed a protein band of approximately 21 kDa but no visible shift of the RhoA protein was seen in rDNT-treated Swiss 3T3 cell lysates (data not shown).

## 5.3 Discussion

This work has shown that rDNT induces the formation of actin stress fibres and focal adhesions, and stimulates DNA synthesis without cell proliferation in Swiss 3T3 cells. Along with other work in our laboratory these results suggest that rDNT modifies the small GTP-binding protein Rho.

Recombinant DNT caused the same morphological changes in EBL cells and Swiss 3T3 cells as wild-type DNT (Garrod, 1994). These changes include binucleation and rounding of cells. DNT is known to be a heat-labile toxin (Munoz, 1971), and when rDNT was heated to 56°C for 30 minutes it did not cause these morphological changes in EBL cells or Swiss 3T3 cells. Recombinant DNT caused binucleation of Swiss 3T3 cells in a dose-dependent manner, with the highest number of binucleated cells at 40 ng ml$^{-1}$ rDNT. The same concentration of rDNT is required to stimulate DNA synthesis optimally in Swiss 3T3 cells.

Unlike CNF (Caprioli *et al.*, 1987; De Rycke *et al.*, 1990), rDNT did not cause multinucleation of cells, even though both toxins activate Rho. It is possible that DNT and CNF modify Rho by different mechanisms, thereby resulting in Rho interacting with different substrates and activating different signalling pathways. Also, rDNT did not cause morphological changes in HeLa cells, confirming the results of Endoh *et al.* (1988a) and Horiguchi *et al.* (1995). It is possible that HeLa cells do not have a receptor for DNT and are therefore unable to take up and process the toxin.

Recombinant DNT drastically modifies the cytoskeleton of Swiss 3T3 cells by stimulating the assembly of actin stress fibres and the formation of focal adhesions, which are regulated by the Rho protein. The effects of rDNT on the cell cytoskeleton were dose-dependent, with as little as 0.16 ng ml$^{-1}$ rDNT stimulating actin stress fibre assembly and focal adhesion formation. Also, rDNT has been shown to stimulate tyrosine phosphorylation of focal adhesion proteins p125$^{FAK}$ and paxillin (Lacerda *et al.*, 1997). The effects of rDNT on the actin cytoskeleton were similar to those observed when a constitutively active mutant of RhoA, [Val$^{14}$] RhoA, was microinjected into Swiss 3T3 cells (Paterson *et al.*, 1990). Many other toxins affect the cell cytoskeleton (see Chapter 1), but only DNT, CNF1 and CNF2 stimulate the formation of actin stress fibres. All other cytoskeletal-acting toxins cause disorganisation of actin in cells. CNF1 and CNF2 cause formation of actin stress fibres

and membrane ruffling (Donelli and Fiorentini, 1992; Oswald *et al.*, 1994), and both toxins have been shown to directly modify the GTP-binding protein Rho (Oswald *et al.*, 1994; Fiorentini *et al.*, 1995).

Initial experiments with sonicated cell extracts showed that rDNT, like wild-type DNT, was capable of stimulating DNA synthesis in quiescent Swiss 3T3 cells. Sonicated cell extracts of DNT1 and B58 stimulated DNA synthesis in a dose-dependent manner, while no stimulation was seen with control extracts. It took about ten times more protein from strain B58 to achieve the same level of [$^3$H]-thymidine incorporation seen in DNT1. Therefore, it can be concluded that the recombinant strain produces approximately ten times more active toxin than B58.

Partially pure rDNT stimulated DNA synthesis in quiescent Swiss 3T3 cells in a dose-dependent manner, at similar concentrations of rDNT required to stimulate the formation of actin stress fibres and focal adhesions. The dose response curve showed that rDNT induced maximal DNA synthesis at approximately 40 ng ml$^{-1}$ (0.28 nM) rDNT. Garrod (1994) found that partially pure wild-type DNT induced maximal DNA synthesis at approximately 70 ng ml$^{-1}$ (0.5 nM) DNT. Horiguchi *et al.* (1993) showed that DNT can stimulate DNA synthesis in a similar cell system with osteoblast-like cells. They found that maximal DNA synthesis was stimulated at 20 pM of pure DNT. These differences may arise from the purity of the DNT and the different cell lines used to assay DNA synthesis. These results show DNT to be a more potent inducer of DNA synthesis than bombesin, a neuropeptide, which stimulated maximal DNA synthesis at 3 nM (Rozengurt and Sinnett-Smith, 1983), but less potent than PMT, which induced maximal stimulation at just 9 pM (Rozengurt *et al.*, 1990). PMT is the most potent mitogen identified for Swiss 3T3 cells and is available in a pure form.

Protein preparations from strains XL1-blue/bluescript and K1310A did not stimulate the formation of actin stress fibres or focal adhesions in Swiss 3T3 cells, and neither stimulated DNA synthesis in quiescent Swiss 3T3 cells. These controls demonstrated that both the cytoskeletal changes and the DNA synthesis activity were due to the rDNT protein and not *E. coli* proteins present in the partially pure preparation. The lack of cytotoxicity of strain K1310A also indicate that the ATP-binding P-loop motif is essential for toxicity and possibly is the location of the active

site of the toxin.

The addition of rDNT to sub-confluent Swiss 3T3 cells prevented cell proliferation, as in serum-starved cells, and the cell number did not increase over 6 days. The concentration of rDNT that prevented cell proliferation, 2.5 ng ml$^{-1}$, was high enough to stimulate binucleation and DNA synthesis in Swiss 3T3 cells. This suggests that rDNT stimulates cells to undergo mitosis, but not to proceed to subsequent cytokinesis. Flow cytometery would be a useful technique to study the stage during the cell cycle at which rDNT blocks cell proliferation. Lacerda *et al.* (1997) showed that rDNT does not induce activation of MAP kinases, p42$^{MAPK}$ and p44$^{MAPK}$, proteins involved in the regulation of cell growth. Therefore rDNT can stimulate DNA synthesis without activation of p42$^{MAPK}$ and p44$^{MAPK}$, suggesting further evidence for a Rho-dependant signalling pathway leading into the S phase of the cell cycle.

Horiguchi *et al.* (1995) reported that Rho proteins in lysates from DNT-treated cells showed a mobility shift by SDS-PAGE. However, the mobility shift of the Rho protein bands varied throughout the experiments. Therefore, I investigated whether rDNT could modify RhoA *in vivo*. Western blots of cell lysates of rDNT-treated cells failed to show that RhoA was modified *in vivo*, which may have been due to the anti-RhoA monoclonal used to screen the lysates. The anti-RhoA monoclonal was against a synthetic peptide corresponding to amino acids 120-150 of RhoA from human cells, it is possible that such a specific monoclonal antibody would not recognise the modified RhoA. It is not know how or where rDNT modifies RhoA.

Many toxins bind to the external surface of the plasma membrane of target cells and are translocated into intracellular compartments (Olsnes *et al.*, 1993). Transient exposure of Swiss 3T3 cells to 10 ng ml$^{-1}$ rDNT for 3 hours stimulated maximal DNA synthesis. This suggests that by 3 hours the toxin was bound to the cells or had entered the cells, and therefore, could not be removed by washing. Horiguchi *et al.* (1993) showed that maximal DNA synthesis was induced after 8 hours transient exposure of 5 ng ml$^{-1}$ DNT to osteoblast-like cells. This difference in transient exposure time may in part be due to the fact that I added twice as much DNT than Horiguchi *et al.* (1993). Rozengurt *et al.* (1990) showed that lower concentrations of PMT required longer preincubation times to induce maximal DNA

synthesis after the removal of unbound toxin. Also, different cell lines were used in these experiments which may have different binding and uptake mechanisms and therefore different exposure times would be required to stimulate DNA synthesis. In cells treated with rDNT, stress fibre formation began at six hours and focal adhesion formation at eight hours. The effect of rDNT on the actin cytoskeleton was slower compared to other molecules such as platelet derived growth factor (PDGF), lysophosphatidic acid (LPA) and bombesin, which have been shown to induce stress fibre formation within 30 minutes (Ridley and Hall, 1992). These results also show that rDNT is a toxin which must be internalised and processed before it can act in the cytosol, unlike PDGF, LPA and bombesin which stimulate the signalling pathways via receptors on the cell surface.

Methylamine is a membrane-permeant weak base which increases endosomal and lysosomal pH. Methylamine can prevent the entry and processing of toxins into the cytoplasm, and is known to block the activity of diphtheria toxin (Mekada et al., 1981), PMT (Rozengurt et al., 1990) and CNF (Oswald et al., 1994). These experiments show that methylamine can block the stimulation of DNA synthesis by rDNT. The results suggest that rDNT is blocked by methylamine, and is therefore taken into the cell through an acidic compartment before acting in the cytosol. This was confirmed by Lacerda et al. (1997), who showed that methylamine inhibited rDNT-induced tyrosine phosphorylation.

Early but not the late addition of methylamine reduced the DNA synthesis of rDNT. This suggested that by two hours rDNT had bound to and entered the cell, reflecting the results obtained from the transient exposure experiments. However, when rDNT and methylamine were added simultaneously DNA synthesis was only reduced by less than 50% of the maximal stimulation. Controls showed that there were high background levels of DNA synthesis due to the methylamine.

The work discussed in this chapter shows that rDNT is translocated into the cytosol of Swiss 3T3 cells, probably via acidic compartments. Once in the cytosol rDNT stimulates cytoskeletal reorganisation, inducing the formation of actin stress fibres and focal adhesions. Recombinant DNT also stimulates DNA synthesis but prevents cell division. The target for rDNT has been shown to be RhoA (G. Pullinger, personnel communication). RhoA is known to regulate the formation of actin stress

fibres and focal adhesions. These results suggest that rDNT modifies Rho to its active form, and this stimulates the activation of the signal transduction pathways regulated by RhoA.

# Chapter 6
## Monoclonal Antibodies Against DNT

## 6.1 Introduction

Until this project, no monoclonal antibodies (mAbs) had been raised against DNT. Evans (1942) produced polyclonal antisera against formaldehyde-treated *B. pertussis* cell extracts in rabbits. This antiserum neutralised the dermonecrotic activity of *B. pertussis* cell extracts as well as *B. parapertussis* and *B. bronchiseptica* cell extracts, suggesting that DNTs from the three *Bordetella* species were antigenically similar. Nakase *et al.* (1969) raised polyclonal antiserum against formaldehyde-treated pure DNT, which neutralised the dermonecrotic activity of DNT, but failed to protect against intracerebral challenge of *B. pertussis* in mice. Since then, several groups have produced neutralising anti-serum against formaldehyde-treated DNT (Livey and Wardlaw, 1984; Endoh *et al.*, 1986c; Horiguchi *et al.*, 1990; Zhang and Sekura, 1991).

Rabbit polyclonal anti-serum was raised in this laboratory against partially purified DNT (Garrod, 1994). Although Western blot analysis showed that the anti-serum recognised DNT, it contained antibodies against other proteins in the partially pure preparation used to immunise the rabbit. Attempts were made to remove by absorption the unwanted antibodies by incubating the anti-serum with extracts from PV6, a DNT-negative *B. bronchiseptica* strain. However, this was unsuccessful because the anti-serum contained antibodies that recognised antigen which were not present in the PV6 cell extracts, and therefore could not be absorbed out against PV6 cell extracts. Also the anti-serum did not neutralise the cytotoxic activity of DNT (Garrod, 1994). Therefore, it was decided to raise monoclonal antibodies against DNT which would be specific to the toxin.

Since the ability to produce stable immunoglobulin-secreting hybridomas was first described by Köhler and Milstein (1975), monoclonal antibodies have become powerful immunochemical tools. The usefulness of mAbs is due to three characteristics: i) their specificity of binding; ii) their homogeneity; and iii) their ability to be produced in unlimited quantities. Also, because hybridomas are single-cell cloned prior to use, mAbs can be produced after immunisation with complex mixtures of antigens. Therefore, partially pure DNT could be used for immunisation, and hybridomas could be screened for the production of specific mAbs against DNT.

Monoclonal antibodies would be useful in several lines of investigation. One main aim was to use mAbs to screen recombinant expression libraries in attempts to

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clone the DNT gene by G. Pullinger in this laboratory, but the DNT gene was cloned and expressed before the mAbs had been produced (Pullinger *et al.* 1996). It was important to obtain neutralising mAbs to DNT for use in cell culture and animal models to assign particular biological activities to DNT. The mAbs could be used for epitope mapping of the protein, as each mAb binds to a specific epitope, enabling the identification of functional domains of the toxin. Also, mAbs are useful tools for immunoaffinity purification and would be useful in the purification of the toxin. Mabs could also be used to stain DNT-treated cells to identify trafficking of the toxin and location of the toxin within the cell. The mAbs were used to screen *B. bronchiseptica* strains isolated from various sources to identify which strains produced DNT and to identify non-cytotoxic mutant rDNT.

## 6.2 Results

### 6.2.1 Detoxification of DNT

Partially purified DNT, containing approximately 6.25 $\mu$g DNT ml$^{-1}$, was dialysed against various concentrations of formaldehyde at room temperature, and then dialysed against PBS to remove remaining formaldehyde from the sample. The cytotoxic effects of DNT on EBL cells were reduced to less than 1% of the original activity when treated with 0.25% or 0.5% formaldehyde for 24 hours (Table 6.1). The cytotoxic effect of DNT was totally lost when dialysed against 1% formaldehyde for 24 hours at room temperature. DNT dialysed against only PBS showed no decrease in cytotoxic activity on EBL cells.

### 6.2.2 Immunisation of mice

Four Balb/c mice were injected subcutaneously with a 1:1 dilution of toxoid DNT (final protein concentration 15 $\mu$g ml$^{-1}$) and Freund's incomplete adjuvant by Brenda Jones. Subsequent injection was given 2 weeks later, and 7 days after this injection a serum sample was obtained from each mouse. The antibody titre of the test serum was determined by ELISA and the serum was also tested on western blots against partially purified DNT to show whether the serum contained antibodies against DNT.

| % Formaldehyde | Cytotoxic activity (1/dilution) |
|---|---|
| 0 | 78125 |
| 0.25 | 125 |
| 0.5 | 5 |
| 1 | 0 |

**Table 6.1** Cytotoxic activity of partial pure DNT on EBL cells after treatment with various concentrations of formaldehyde for 24 hours at room temperature. All samples were tested in duplicate and diluted to an end point.

| Mouse Serum | ELISA Titre at $OD_{450}$ = 0.2 |
|---|---|
| Normal | 1/100[1] |
| Pooled | 1/25600 |
| Mouse 1 | 1/12800 |
| Mouse 2 | 1/25600 |
| Mouse 3 | 1/12800 |
| Mouse 4 | 1/25600 |

**Table 6.2** Comparison of the ELISA titre of the post injection serum from the four mice immunised with partially pure DNT.

[1] The highest concentration of each serum sample used in this assay was 1/100; at this dilution the normal mouse serum did not give an OD reading above the ELISA end point.

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### 6.2.3 Development of ELISA

An ELISA was developed to screen the hybridoma tissue culture supernatants for antibodies against *B. bronchiseptica* antigens. Firstly the concentration of antigen required to coat the ELISA plates was determined. The highest spectrophotometric readings were obtained from the wells with the highest concentrations of antibody and antigen. For each antibody dilution with a particular antigen concentration, an approximately sigmoidal decrease in colour intensity was seen. The lowest antigen concentration to give a strong colour intensity and complete sigmoid titre curve was chosen.

An antigen dilution of 1 in 50 (total protein concentration 1 $\mu$g ml$^{-1}$) was chosen to coat the ELISA plates for future assays. Controls with normal mouse serum, i.e. from mice not exposed to DNT, showed that high serum concentrations gave a strong colour intensity (Figure 6.1). Different blocking strategies were investigated to prevent this non-specific binding. Blocking for 3 hours with 3% (w/v) BSA, 5% (w/v) BSA or 1% (w/v) gelatin did not prevent this non-specific binding of control serum. Blocking for 3 hours with 20% (w/v) marvel/PBS reduced the non-specific binding of control serum, except at very high serum concentrations. The addition of 2% (w/v) marvel in the test sera prevented all non-specific binding (Figure 6.2). For this ELISA system, an end point of $OD_{450} = 0.2$ was chosen. Optical density readings above this value were regarded as positive, while those below were taken as negative ELISA results.

The sera from all four hyperimmune mice gave a strong antibody response as determined by ELISA (Table 6.2). Western blot analysis of the test sera showed that mice 2, 3, and 4 were producing antibodies against DNT, but that mouse 1 was not (data not shown). Mice 2 and 4 were chosen for the development of mAbs.

### 6.2.4 Production of monoclonal antibodies

Hybridomas were prepared by Brenda Jones. Fusion F564 of mouse 2 gave thirty-three positive hybridoma tissue culture supernatants from the 860 supernatants screened by ELISA. These supernatants contained antibodies that recognised antigen from partially pure DNT preparations. These positive tissue culture supernatants were then screened against partially pure DNT by Western blot. Two of the mAbs
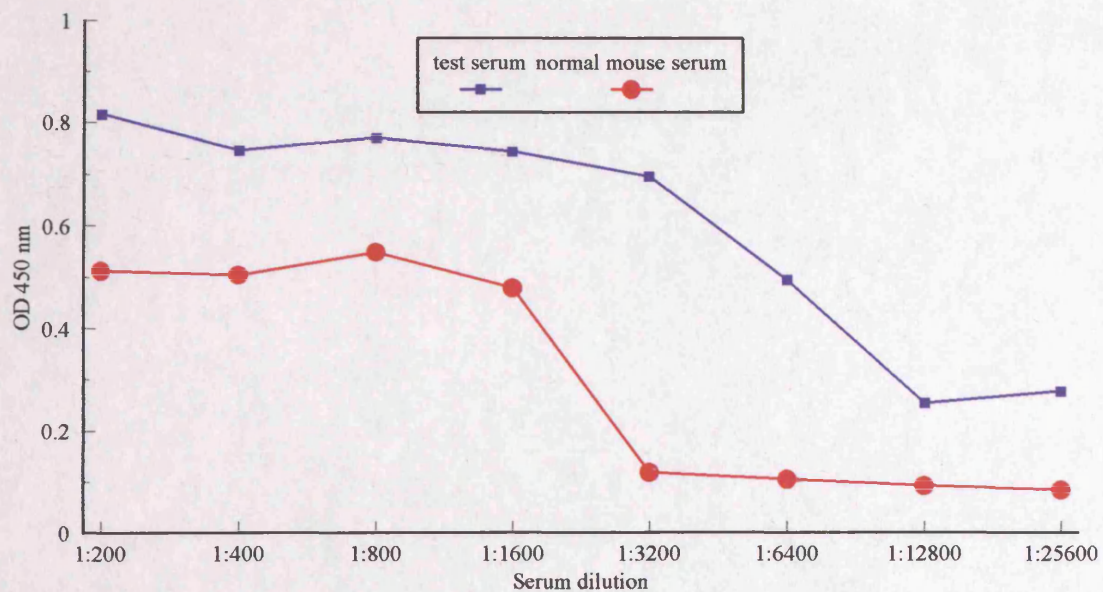
**Figure 6.1** ELISA titration curve of pooled antisera from four mice immunized with partially pure detoxified DNT, and normal mouse serum. The ELISA was blocked with 3% (w/v) BSA for 2 hours.
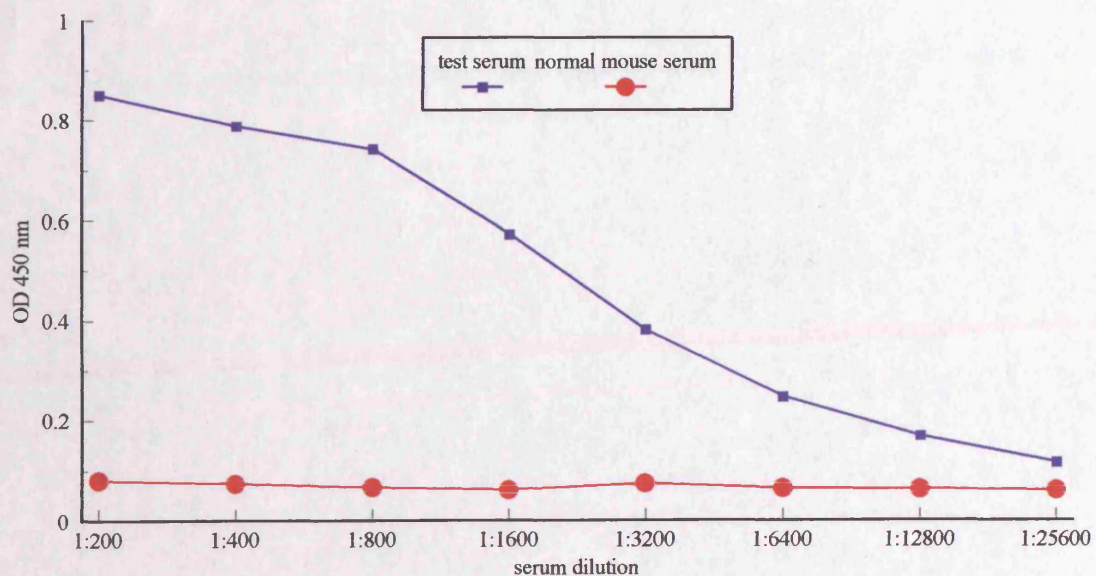


**Figure 6.2** ELISA titration curve of pooled antisera from four mice immunized against partially pure detoxified DNT, and normal mouse seum. The ELISA was blocked with 20% (w/v) Marvel for 3 hours and 2% (w/v) Marvel was included with the screened sera.

recognised a 145 kDa protein, DNT. Both mAbs were immunoglobulin isotype IgG1. The cell lines were named F564 (AE9) and (BG3).

Fusion F571 of mouse 4 gave thirty eight positive hybridoma tissue culture supernatants from the 770 supernatants screened by ELISA. These positive tissue culture supernatants were screened by Western blot, and only one of the mAbs recognised DNT. This cell line was named F571 (EG3).

All three mAbs were purified from the tissue culture supernatants by a protein G-Sepharose column (Pharmacia) (data not shown).

### 6.2.5 Specificity of monoclonal antibodies for DNT

The mAbs AE9 and BG3 recognised a 145 kDa band in sonicated cell extracts of *B. bronchiseptica* B58 (Figures 6.3a and 6.3b), and a 145 kDa band in partially purified DNT (Figures 6.4a and 6.4b). Monoclonal antibody EG3 did not recognise any antigens in sonicated cell extracts of B58, but did bind to a 145 kDa protein in partially pure preparations of DNT (Figures 6.3c and 6.4c). None of the mabs recognised any proteins in sonicated cell extracts of *B. bronchiseptica* PV6 (Figure 6.3).

All the mAbs recognised a 145 kDa protein band in DNT1 and partially purified fractions of rDNT (Figures 6.3 and 6.4). The mAbs did not recognise any antigens in sonicated cell extracts of *E.coli* XL1-blue/bluescript (Figure 6.3). MAbs AE9 and BG3 recognise several other proteins of about 50 and 66 kDa in sonicated cell extracts of DNT1, and these proteins were sometimes present in purified rDNT samples.

Monoclonal antibody AE9 recognised a 145 kDa protein in sonicated cell extracts of K1310A and the purified fractions of K1310A (Figure 4.18). MAbs BG3 and EG3 were not screened against K1310A samples.

Monoclonal antibody AE9 recognised a 145 kDa protein from sonicated cell extracts of two *B. pertussis* phase 1 strains, Tohama (DCH8A) and Wellcome 28 (W28G) (Figure 6.5). The *B. pertussis* strains did not grow in liquid media, Hornibrooks media or Stainer and Schölte media, so colonies were scraped off Bordet-Gengou plates into PBS for sonication.
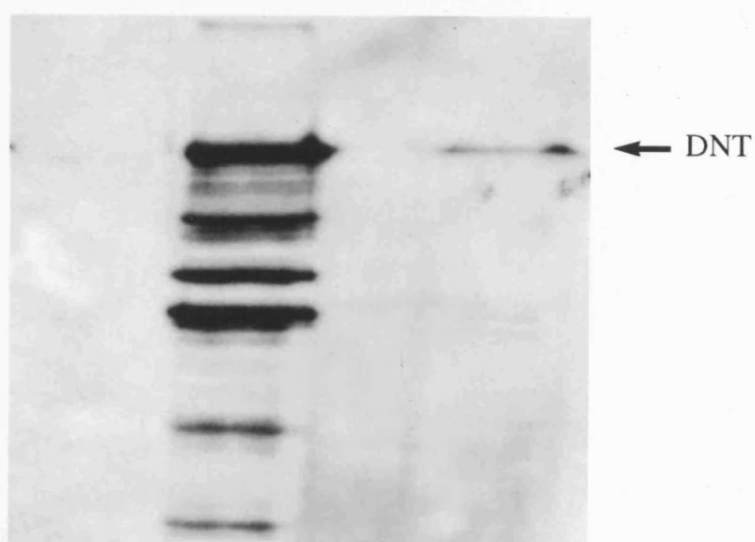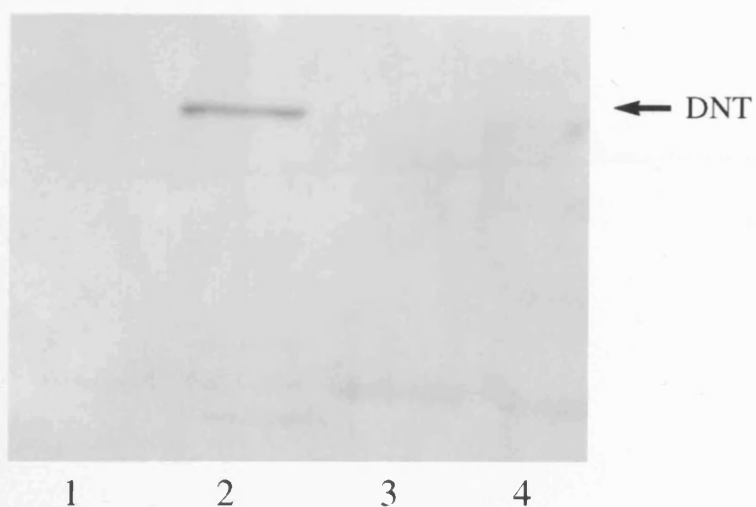
**Figure 6.3** Western blot of sonicated cell extracts of strains XL1blue/bluescript, PDNT1, and *B. bronchiseptica* PV6 and B58 probed with anti-DNT mAbs. A, mAb AE9; B, mAb BG3; C, mAb EG3; all mAbs were used at 1/100 dilution.
Lane 1, XL1blue/bluescript; lane 2, PDNT1; lane 3, PV6; lane 4, B58.
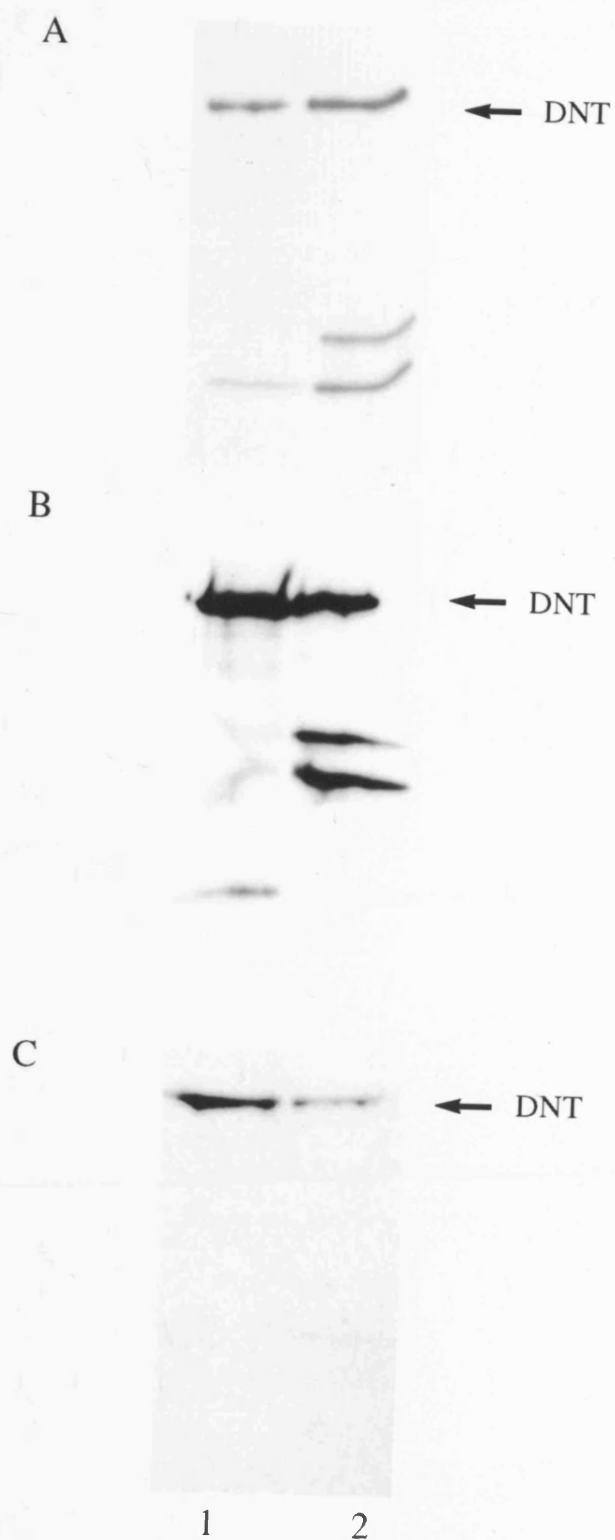
**Figure 6.4** Western blot of partially pure wild-type DNT and rDNT probed with anti-DNT mAbs. A, mAb AE9; B, mAb BG3; C, mAb EG3; all mAbs were used at 1/100 dilution. Lane 1, wild-type DNT and lane 2, rDNT.
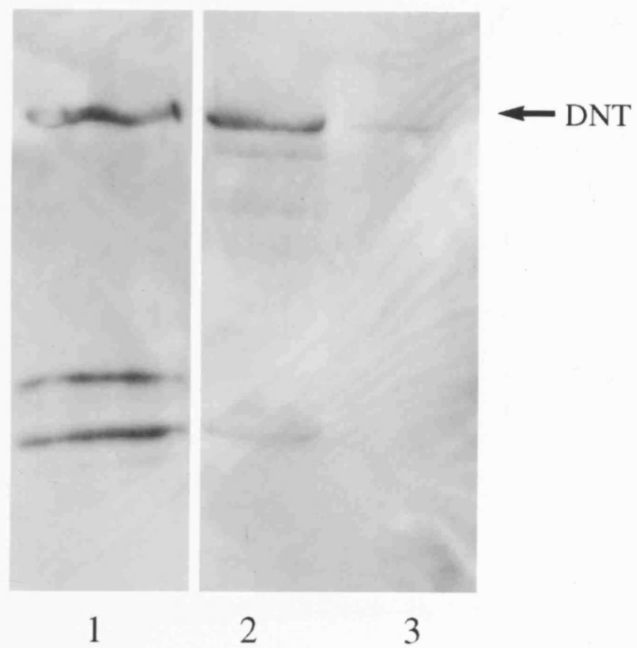
**Figure 6.5** Western blot of sonicated cell extracts of *B. pertussis* probed with anti-DNT mAb AE9.
Lane 1, partially pure rDNT; lane 2, *B. pertussis* Wellcome 28; lane 3, *B. pertussis* Tohama.

### 6.2.6 Neutralisation of cytotoxic effect of DNT on EBL cells by monoclonal antibodies

The addition of the mAbs did not inhibit the cytotoxic effect of partially pure DNT on EBL cells, even at a 1160:1 ratio of mAb to protein.

### 6.2.7 Biotinylation of monoclonal antibodies

Monoclonal antibody BG3 was biotinlylated with BAC-sulfoNHS (biotinamidocaproate N-hydroxysuccinimide ester) (Sigma) at various molar ratios. Western blot analysis showed that a 10:1 biotin to mAb ratio gave the most effective biotin labelling. Monoclonal antibodies AE9 and EG3 were labelled with biotin using a 1:10 molar ratio of BAC-sulfoNHS to mAb. Western blot analysis showed that the biotinylated mAbs recognised partially purified DNT and rDNT (Figure 6.6).

### 6.2.8 Comparison of monoclonal antibody binding sites

To investigate if any of the mAbs recognised the same region of the toxin a competition ELISA was performed. The biotinylated mAb was competed against unlabelled self or unlabelled other mAb. Unfortunately, no results were obtained from the competition assays. The results from the controls suggested that the mAbs were no longer biotinylated and did not bind to the strepavidin-HRP conjugate.

### 6.2.9 Western blot analysis of EBL cells and Swiss 3T3 cells

To investigate if the mAbs could be used to study the trafficking of DNT and the location of DNT within cells, cell extracts of EBL cells and Swiss 3T3 cells were prepared and analysed by Western blot. None of the three mAbs recognised any proteins in the cell extracts (data not shown).

### 6.2.10 Screening B. bronchiseptica strains from different sources

Twenty-six B. bronchiseptica strains were provided by N. Guiso, (Unité de Bactériologie Moléculaire et Médicate, Institut Pasteur, Paris). The strains were isolated from different sources: dogs, pigs, rabbits, horses and humans. Sonicated cell extracts from 10 ml overnight cultures were analysed by Western blot and on EBL cells (Table 6.3). Monoclonal antibody AE9 was used to screen the strains because it
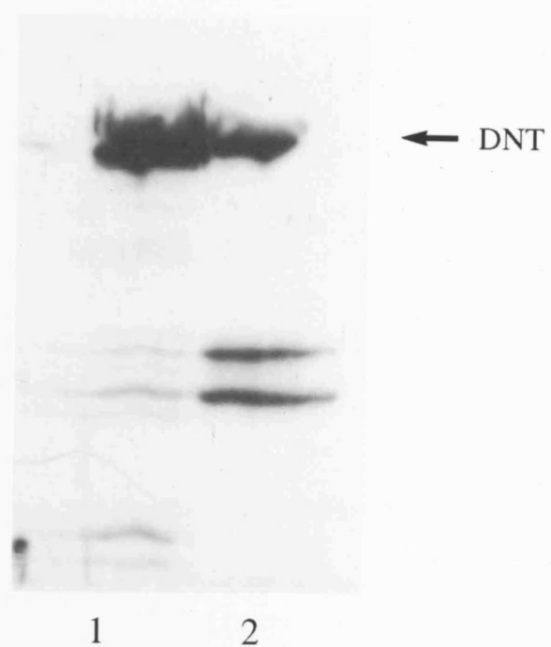
**Figure 6.6** Western blot of partially pure wild-type DNT and rDNT probed with biotinylated anti-DNT mAb BG3.
Lane 1, wild-type DNT and lane 2, rDNT.

bound most strongly to DNT, as visualised by Western blot. *B. bronchiseptica* B58 was used as the DNT-positive control, and PV6 was used as the DNT-negative strain.

None of the cell extracts from five rabbit *B. bronchiseptica* strains had a 145 kDa protein that was recognised by mAb AE9, and none was cytotoxic to EBL cells. Two of the four strains isolated from dogs were cytotoxic to EBL cells, and showed a 145 kDa protein by Western blot analysis. Three of the six pig isolates showed a 145 kDa protein recognised by mAb AE9 and were cytotoxic to EBL cells. Two of the pig strains, Bg1 and 374, had no protein recognised by mAb AE9, but were slightly cytotoxic to EBL cells. Three of the six strains isolated from humans had a 145 kDa protein recognised by mAb AE9 and were cytotoxic to EBL cells, whilst two others, VAL and ALI, had neither of these characteristics. However, the human isolate SEI had no protein recognised by mAb AE9, but was cytotoxic to EBL cells to an end point dilution of 1/125, the same as the positive control strain B58. Three of the strains isolated from horses were cytotoxic to EBL cells and showed a 145 kDa band by Western blot analysis. The other horse isolate had no 145 kDa protein recognised by AE9, but was slightly cytotoxic to EBL cells.

| B. bronchiseptica strains | Western blot anti-DNT mAb AE9 | Cytotoxicity end point dilution |
|---|---|---|
| **Dogs** | | |
| 286 | - | - |
| 11 | ++ | 1/125 |
| 335 | + | 1/125 |
| CIP 52125 | - | - |
| **Rabbits** | | |
| 9.73 | - | - |
| LC2 | - | - |
| LAPR | - | - |
| CIP 55110 | - | - |
| LC3 | - | - |
| **Pigs** | | |
| B58 | ++ | 1/125 |
| PV6 | - | - |
| 46093 | + | 1/25 |
| 12 | ++ | 1/125 |
| 5 | + | 1/25 |
| 374 | - | 1/5 |
| Bg1 | - | 1/5 |
| **Humans** | | |
| DEL | + | 1/25 |
| DANG | - | 1/25 |
| VAL | - | - |
| ALI | - | - |
| R1 | ++ | 1/125 |
| SEI | - | 1/125 |
| **Horses** | | |
| CVE | - | 1/25 |
| CVH | + | 1/25 |
| CV1 | + | 1/25 |
| CV2 | + | 1/25 |

**Table 6.3** Screening *B. bronchiseptica* strains for DNT with anti-DNT mAb AE9 and for cytotoxic activity on EBL cells.

## 6.3 Discussion

In this work, three mAbs, AE9, BG3 and EG3, were raised against DNT from *B. bronchiseptica* B58. Although polyclonal antibodies have been raised against DNT from *B. pertussis* and *B. bronchiseptica*, this is the first time mAbs have been raised to DNT.

Detoxification of partially pure DNT was necessary before the mice could be immunised with it. Several methods have been used to detoxify DNT. Garrod (1994) used heat treated DNT to raise polyclonal antibodies in rabbits, but the resulting antiserum did not neutralise the cytotoxic activity of DNT. Evans (1942) also failed to produce neutralising antibodies to the dermonecrotic activity of the toxin when using heat-inactivated cell extract from *B. pertussis*. Chemical treatment has been used successfully by several groups to produce detoxified DNT for antiserum production (Nakase *et al.*, 1969; Livey and Wardlaw, 1984; Endoh *et al.*, 1986c; Horiguchi *et al.*, 1990). Horiguchi *et al.* (1990) detoxified DNT using 0.4% formalin for 5 days at 37°C, whereas Livey and Wardlaw (1984) found that 0.25% formaldehyde detoxified DNT in 1 day at 37°C, but at 4°C, 0.25% formaldehyde took 9 days to detoxify DNT. In order to produce neutralising mAbs, formaldehyde was used to detoxify DNT and several concentrations were tested. Room temperature was used for detoxification, because toxin activity would be lost at 37°C due to heat and toxin could lost due to long dialysis at 4°C. For immunisation of mice DNT was detoxified by dialysis against 1% formaldehyde for 24 hours at room temperature.

A rapid and simple ELISA was developed for the screening of tissue culture supernatants from the hybridoma cell fusions. It was necessary to block with 20% (w/v) marvel/PBS, and 2% (w/v) marvel in the test serum to prevent non-specific binding.

Mice 2 and 4 were used for the development of mAbs because they had the highest antibody response as determined by ELISA, and produced anti-DNT antibodies as shown by Western blot analysis. Three hybridomas were identified that produced specific mAbs against DNT.

The purified mAbs recognised DNT from *B. bronchiseptica* B58, and rDNT from strain DNT1. Monoclonal antibody EG3 recognised purified DNT, but bound less strongly to DNT compared to the other mAbs. Therefore, binding was not seen in B58

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sonicated cell extracts due to the relatively small quantity of DNT produced by the bacteria. Monoclonal antibodies AE9 and BG3 also recognised several other protein bands in sonicated cell extracts of DNT1. Neither AE9 nor BG3 recognised these proteins in sonicated cell extracts of XL1-blue/bluescript, therefore the proteins may be breakdown products of rDNT which were not recognised by EG3. If rDNT was being degraded by proteases it should appear as a smear of protein bands. Possibly rDNT is cleaved at a certain point, or rDNT is not being completely translated by *E. coli*.

Monoclonal antibody AE9 recognised the inactive, mutated rDNT produced by strain K1310A, and also recognised DNT from two strains of *B. pertussis*. The DNTs from three *Bordetella* species were thought to be serologically similar, because antibodies against *B. pertussis* DNT neutralised the dermonecrotic activity of *B. bronchiseptica* and *B. parapertussis* (Evans, 1940). The recent sequencing of the *B. pertussis* and *B. bronchiseptica* DNT genes (Walker and Weiss, 1994; Pullinger *et al.*, 1996) shows that the nucleotide sequences are more than 99% identical and there are only 11 amino-acid differences between the two DNTs. Therefore, it is not surprising that the mAbs against *B. bronchiseptica* DNT recognised DNT from *B. pertussis*.

None of the three mAbs neutralised the cytotoxic activity of DNT on EBL cells, possibly because they do not recognise epitopes involved in the activity of the toxin, or because that formaldehyde-treated antigen resulted in antibody unable to recognise the native protein. Formaldehyde-treated toxin resulted in the production of anti-DNT neutralising polyclonal antibodies in rabbits (Nakase *et al.*, 1969; Livey and Wardlaw *et al.*, 1984; Endoh *et al.*, 1986c; Horiguchi *et al.*, 1990). However, Livey and Wardlaw (1984) failed to raise anti-DNT neutralising antibodies in mice, despite using several different immunisation conditions. They found that the same formaldehyde-treated DNT induced the formation of anti-DNT neutralising antibodies in rabbits. The production of a large bank of mAbs against DNT identify a mAb that binds to an epitope involved in activity and that does neutralise toxin activity. Immunisation of the mice with detoxified DNT, followed by immunisation with active DNT, may be necessary to raise neutralising antisera against the native protein. However, DNT has been reported to suppress *in vivo* antibody production in mice due to splenoatrophy (Horiguchi *et al.*, 1992), so immunisation with active DNT may not

be an effective method. The production of polyclonal antibodies in rabbits seems the best method for producing neutralising antibodies, especially if DNT produces a poor immunogenic response in mice.

It is possible that the mAbs do recognise the epitopes involved in the activity of DNT. However, they do not neutralise the toxin activity *in vivo* because the mAbs are removed during the uptake of the toxin into the cell through acidic compartments. Therefore, it would be interesting to investigate whether the mAbs could inhibit the *in vitro* modification of Rho by rDNT.

A competition ELISA was performed to compare the antibody binding sites of the three mAbs. All the mabs were labelled with biotin. Western blot analysis showed that the mAbs were labelled and recognised DNT. Unfortunately no results were obtained from the competition assays. The results from the controls suggested that the mAbs were no longer biotinylated and not binding to the strepavidin-HRP conjugate. There was insufficient time to relabel the mAbs and repeat the competition assays.

Twenty-six strains of *B. bronchiseptica* isolated from various sources were screened by Western blot with anti-DNT monoclonal AE9 for the production of DNT. Eleven of the strains produced DNT and were cytotoxic to EBL cells. These results correspond with the published results that show dog isolate 11, and human isolates DEL and R1 produced DNT, as demonstrated by the dermonecrotic activity assay in suckling mice (Gueirard and Guiso, 1993; Gueirard *et al.*, 1995). Gueirard and Guiso (1993) also showed that rabbit isolates 9.73 and LAPR did not produce DNT. Five of the isolates showed no DNT production by Western blot analysis, but were cytotoxic to EBL cells. Gueirard and Guiso (1993) showed that one of these isolates, SEI, did not produce DNT but did produce other virulence factors such as AC/HA, FHA and pertactin. Southern blot analysis showed that SEI did not hybridise to a DNT probe so does not have the DNT gene (G. Pullinger, personnel communication). Therefore, the cytotoxic activity on the EBL cells maybe due to other virulence factors. This implies that the EBL cell assay is sensitive to other factors, not just DNT, and that screening the isolates with specific antibodies against DNT is more accurate than using the EBL cell assay.

These results show that there is no correlation between the detection of DNT with specific antibodies and lethality in intranasal infection of mice (N. Guiso,

personnel communication). Rabbit isolates 9.73 and LAPR had no detected DNT and had $LD_{50}$ of 3 x $10^6$ and 2 x $10^6$ CFU respectively (Gueirard and Guiso, 1993), whereas, dog isolate 11 and human isolate R1 produced DNT and had $LD_{50}$ of 2 x $10^6$ and 1 x $10^8$ CFU respectively (Gueirard and Guiso, 1993, Gueirard et al., 1995). However, human isolate DEL had low levels of DNT detected and had a $LD_{50}$ of 5 x $10^3$ CFU (Gueirard and Guiso, 1993). Therefore, the expression of DNT or the level of DNT production does not play an important role in the initiation of infection in the murine model. DNT may play a role later in infection when bacterial cells are lysed and DNT is released. DNT may be more important in the infection of different animals. DNT plays an important role in atrophic rhinitis of pigs and four of the pig isolates produced DNT. However, DNT may not be important in infection of rabbits because none of the rabbit isolates produced DNT.

These anti-DNT mAbs have many applications as part of future research. For example, mAbs could be used to affinity purify DNT, especially from Bordetella as there is no cross-reaction with other proteins in the cell sonicate. Monoclonal antibodies AE9 and BG3 would be less useful for the purification of rDNT from DNT1, as they recognise several other proteins in the cell sonicate. The mAbs could also be used to investigate the trafficking of the toxin and location of the toxin within cells, since none of the mAbs cross reacts with proteins in cell extracts of either EBL cells or Swiss 3T3 cells.

*Chapter 7*

*General Discussion*

The *Bordetella* dermonecrotic toxin (DNT) is produced by all the *Bordetella* species, and in each species it has similar biological activities and immunological properties. DNT is considered to be an important virulence factor in atrophic rhinitis of pigs. However, its role in atrophic rhinitis, and also human disease, is poorly understood due to difficulties in its purification and the absence of cloned rDNT. Recently, the DNT gene from *B. bronchiseptica* was cloned and expressed in *E. coli* (Pullinger *et al.*, 1996).

In this thesis a method was developed for the partial purification of rDNT, based on the purification method developed by Garrod (1994) for wild-type DNT. These preparations of rDNT were used to investigate its *in vitro* effects. The evidence that DNT was the active component in these preparations is very strong because good negative controls were available, i.e. partially pure, inactive, mutant rDNT from K1310A, and preparations from XL1-blue/bluescript. Three monoclonal antibodies (mAbs) were produced against *B. bronchiseptica* DNT, which also recognised rDNT from strain DNT1. These mAbs could be used to purify DNT by affinity chromatography, thereby improving the purification of DNT from sonicated cell extracts.

My work showed that rDNT, like wild-type DNT, was cytotoxic to EBL cells and caused binucleation of Swiss 3T3 cells. Recombinant DNT stimulated DNA synthesis in quiescent Swiss 3T3 cells to the same extent as wild-type DNT, and inhibited subsequent cell proliferation. Furthermore, rDNT induced the rearrangement of the actin cytoskeleton of Swiss 3T3 cells, resulting in the formation of actin stress fibres and focal adhesions.

Work in this laboratory has shown that rDNT modifies the small GTP-binding protein Rho (G. Pullinger, unpublished). Horiguchi *et al.* (1995) suggested that wild-type DNT modified Rho in an osteoblast cell line. Lacerda *et al.* (1997) have shown that rDNT activates Rho-stimulated tyrosine phosphorylation of focal adhesion proteins, p125$^{FAK}$ and paxillin. These results suggest that Rho is the target for DNT, and together with my results suggest that DNT modifies Rho to its active form.

Identification of Rho as the target of DNT, and the stimulation of the Rho signalling pathways by DNT, provide an explanation for the biological activities of DNT. DNT acts specifically on smooth muscle cells *in vitro* (Endoh *et al.*, 1988a;

Nagai *et al.*, 1992a). Endoh *et al.* (1986a; 1986b) put forward the hypothesis that DNT-induced dermonecrotic lesions were caused by constriction of smooth muscle cells, resulting in reduced blood supply to affected areas. Activated Rho is involved in phosphorylation of the myosin light-chain (MLC) phosphatase, which leads to the stimulation of actin stress fibre formation and contraction of smooth muscle cells. Therefore, DNT may stimulate this signalling pathway through activation of Rho, leading to the characteristic dermonecrotic lesions.

Rho is involved in the regulation of cell growth and stimulation of cell cycle progression. DNT-activation of Rho may induce DNA synthesis, but the pathways used by Rho to stimulate DNA synthesis are unknown. There is evidence that Rho is involved in the regulation of cytokinesis, but its role remains unclear (Mabuchi *et al.*, 1993; Kishi *et al.*, 1993). Cytoplasmic cell division requires the formation of a contractile ring composed of actin and myosin filaments (Fishkind and Wang, 1995). DNT may inhibit cytokinesis by sequestering the actin filaments into stress fibres, making them unavailable for assembly of the contractile ring. Alternatively, DNT may alter regulation of the S phase/mitosis transition. Normally, complete mitosis is a prerequisite for the start of the next S phase, however, there are specific examples of genetic control when a cell is able to undergo DNA synthesis without cytoplasmic division. For example, the ts41 temperature sensitive mutant in CHO cells leads to successive replication of DNA without cellular division (Handeli and Weintraub, 1992). The sequence of cell cycle events is controlled by many cyclins and cyclin-dependent kinases, which play an important role in the stringent S phase/mitosis regulation (Wuarin and Nurse, 1996). Rho and its family members also regulate signalling pathways that lead to mitogensis (Vojtek *et al.*, 1995; Olson *et al.*, 1995). DNT may alter the sequence of cell cycle events via the Rho signalling pathways. This inhibition of cytokinesis by DNT explains the *in vivo* role for DNT-inhibition of osteoblast formation in the pathogenesis of atrophic rhinitis (Kimman *et al.*, 1987; Horiguchi *et al.*, 1993).

*Bordetella* species have been shown to invade and survive inside mammalian epithelial cells (Ewanowich *et al.*, 1889a; 1989b; Savelkoul *et al.*, 1993; Schipper *et al.*, 1994). The virulence factors which facilitate this intracellular entry and survival have not been clearly identified. However, DNT may promote the phagocytic uptake

of *Bordetella* species by the activation of Rho. Actin microfilaments are required for phagocytosis. Entry of *Shigella* species into epithelial cells is Rho-dependent, and Rho was involved in actin polymerisation induced by *Shigella* species (Adam *et al.*, 1996). CNF1 induces phagocytic-like activity in epithelial cells, which allows uptake of CNF1-producing *E. coli* inside membrane bound vacuoles, and may allow bacteria to survive and multiply intracellularly *in vivo* (Falzano *et al.*, 1993; Donelli *et al.*, 1994). Therefore, it is possible that activation of Rho by DNT, and stimulation of actin stress fibre formation may induce the phagocytic uptake of *Bordetella* species in epithelial cells.

It is proposed that PMT, CNF1, CNF2 and DNT are member of the same toxin family, based on sequence homology and a number of shared properties. They are all high molecular weight, single chain polypeptides which are not secreted by the bacteria. All these toxins induce dermonecrosis, are lethal to mice, and cytotoxic to cultured cells. My research provides further evidence that DNT should be considered a member of this family of necrotizing toxins.

All these necrotizing toxins stimulate DNA synthesis in quiescent cells. Stimulation of DNA synthesis by PMT in quiescent Swiss 3T3 cells leads to cell proliferation (Rozengurt *et al.*, 1990). However, DNT and the CNFs induce nuclear division without subsequent cell division (Fiorentini *et al.*, 1988; Horiguchi *et al.*, 1993; Oswald *et al.*, 1994; this work). This leads to the development of large bi- or multi-nucleated cells. All these toxins have been shown to stimulate the formation of actin stress fibres and focal adhesions (Fiorentini *et al.*, 1988; Oswald *et al.*, 1994; Horiguchi *et al.*, 1995; Lacerda *et al.*, 1996; this work), and stimulate tyrosine phosphorylation of focal adhesion proteins p125$^{FAK}$ and paxillin (Lacerda *et al.*, 1996; 1997). Recently it has been shown that both the CNFs and DNT target and modify the Rho protein involved in the regulation of the cell cytoskeleton (Oswald *et al.*, 1994; Fiorentini *et al.*, 1995; Horiguchi *et al.*, 1995; G. Pullinger, unpublished). Indirect evidence suggests that PMT activates $G_q$ (Murphy and Rozengurt, 1992), and recently PMT has been shown to activate the inositol phosphate signalling pathway via $G_{q\alpha}$ (Wilson *et al.*, 1997). In addition, the effect of PMT on the cell cytoskeleton implies that the GTP-binding proteins of the Rho family are involved (Lacerda *et al.*, 1996). Therefore, all of these toxins modify GTP-binding proteins, but the mode of

modification is unknown. The ATP-binding motif of DNT is essential for activity, and so the modification of Rho by DNT may involve ATP hydrolysis as a source of energy.

I have also shown that DNT, like PMT and CNF, appears to enter the cytoplasm of the target cell via acid compartments (Rozengurt *et al.*, 1990; Oswald *et al.*, 1994). However, the cell surface receptor of these toxins is probably different because CNF causes multinucleation of HeLa cells (Caprioli *et al.*, 1987; De Rycke *et al.*, 1990), while DNT did not change the morphology of HeLa cells.

The partial purification of rDNT and inactive mutant rDNT has allowed the characterisation of the effects of the toxin on cells. Further studies of this toxin, including analysis of its enzymatic mode of action, its modification of Rho, and its effects *in vivo*, will provide valuable information about the role of DNT in disease. Also, DNT may be a useful tool for analysis of cell responses involving the important signalling molecule Rho.

*Appendices*

## Appendix A - Media

All media were autoclaved for 20 min at 121°C, 15 lb in$^{-2}$ on a liquid cycle.

### Modified Hornibrook medium

    5 g sodium chloride

    0.2 g potassium chloride

    0.25 g dipotassium hydrogen orthophosphate

    0.25 mg calcium chloride dihydrate

    10 g casamino acids (Difco)

    1 g soluble starch (Difco)

    1 mg nicotinamide (Sigma)

    10 ml of 0.1% (v/v) glutathione solution

The solutes were dissolved and adjusted to pH 7.0 with HCl. The volume was made up to 990 ml with distilled water and the medium sterilised. The glutathione (10 ml) was sterilised by filtration and added after autoclaving.

### Bordet-Gengou Agar

    30 g Bordet-Gengou agar base (Difco)

    10 g peptone (Difco)

    10 ml glycerol

    15% (v/v) horse blood (Oxoid)

The solutes were dissolved in distilled water and the volume made up to 850 ml. The medium was sterilised, allowed to cool to 50-60°C and 150 ml of the sterile horse blood was added. Plates were poured using 20 ml of medium per plate.

### Stainer and Schölte medium

    240 mg L-proline

    670 mg sodium glutamate

    40 mg L-cystine

    2.5 g sodium chloride

    500 mg potassium dihydrogen orthophosphate

    200 mg potassium chloride

    100 mg magnesium chloride hexahydrate

20 mg calcium chloride

10 mg iron(II) sulphate heptahydrate

6.075 g Tris base

20 mg ascorbic acid

4 mg niacin

100 mg glutathione

The solutes were dissolved and adjusted to pH 7.6 with 2.5 M HCl. The volume was made up to 1 litre with distilled water and the medium sterilised. The cystine, glutathione and niacin were sterilised by filtration and added after autoclaving.

**Peptone Water**

10 g peptone

5 g sodium chloride

The solutes were dissolved in distilled water, and the volume made up to 1 litre and sterilised.

**Blood Agar**

5% (v/v) horse blood was added to blood agar base (Oxoid) and poured into plates.

**Luria-Bertani (LB) medium**

10 g Bacto-tryptone (Difco)

5 g Bacto-yeast extract (Oxoid)

10 g sodium chloride

The solutes were dissolved, and adjusted to pH 7 with 5 M NaOH. The volume was made up to 1 litre with distilled water and the medium sterilised. For LB agar, 1.5% (w/v) of bacto-agar (Oxoid) was added before sterilisation.

**2xYT medium**

16g Bacto-tryptone (Difco)

10g Bacto-yeast extract (Oxoid)

5g sodium choride

The solutes were dissolved, and adjusted to pH 7 with 5 M NaOH. The volume was made up to 1 litre with distilled water and the medium sterilised.

## *Appendix B - SDS-PAGE solutions*

A 40% (w/v) stock solution of 29:1 acrylamide: bis-acrylamide (Sigma) was used. The solution was filtered (0.45 µm) before use and stored at 4°C.

**Resolving gel**

    5% or 8% (v/v) acrylamide/bis acrylamide stock solution

    375 mM Tris/HCl, pH 8.8

    0.1% (w/v) SDS

    0.02% (w/v) ammonium persulphate

    0.1% (v/v) N, N, N', N'- Tetramethylethylenediamine (TEMED) (BioRad)

**Stacking gel**

    4% (v/v) acrylamide/bis acrylamide stock solution

    125 mM Tris/HCl, pH 6.8

    0.1% (w/v) SDS

    0.05% (w/v) ammonium persulphate

    0.1% (v/v) TEMED

**Running buffer**

    25 mM Tris

    192 mM glycine (Gibco BRL)

    0.1% (w/v) SDS

**Sample buffer**

    62.5 mM Tris/HCl, pH 6.8

    2% (w/v) SDS

    10% (v/v) glycerol

    5% (v/v) β mercaptoethanol

    0.005% (w/v) bromophenol blue

All buffers were made up to the required volume with distilled water.

## Appendix C - Molecular weight markers

| Protein | Molecular weight (kDa) |
|---|---|
| Myosin | 205 |
| β-Galactosidase | 116 |
| Phosphorylase B | 97 |
| Albumin (bovine) | 66 |
| Albumin (egg) | 45 |
| Carbonic anhydrase | 29 |

Molecular weight protein markers (SDS-6H, Sigma) used for SDS-PAGE.

| Protein | Molecular weight (kDa) |
|---|---|
| $\alpha_2$-Macroglobulin | 190 |
| β-Galactosidase | 108 |
| Fructose-6-phosphate kinase | 84 |
| Pyruvate Kinase | 67 |
| Fumarase | 55 |
| Lactic dehydrogenase | 39 |
| Triosephosphate isomerase | 35 |

Prestained molecular weight protein markers (SDS-7B, Sigma) used for SDS-PAGE and protein transfer.

# *Appendix D - staining solutions*

**Silver staining solutions**

> Fixing solution
>> 50% (v/v) ethanol
>>
>> 10% (v/v) acetic acid
>
> Incubation solution
>> 30% (v/v) ethanol
>>
>> 800 mM sodium acetate
>>
>> 8 mM sodium thiosulphate ($5H_2O$)
>>
>> 0.5% (v/v) glutaraldehyde (25% w/v)
>
> Silver solution
>> 6 mM silver nitrate
>>
>> 0.02% (v/v) formaldehyde
>
> Developing solution
>> 236 mM sodium carbonate
>>
>> 0.01% (v/v) formaldehyde
>
> Stop solution
>> 39 mM EDTA

All solutions were made up to the required volume with distilled water.

**EBL stain**

> 0.2% (w/v) crystal violet (Searle Diagnostics) and 2% (v/v) acetic acid
>
> 12% (v/v) neutral buffered formalin

Crystal violet was dissolved in acetic acid and the solution passed through a 0.45 μm filter and stored at room temperature. The stain was made up fresh for each use by mixing equal volumes of the crystal violet solution and the neutral buffered formalin.

**Ponceau S**

> 0.2% (w/v) ponceau S (Sigma)
>
> 3% (w/v) trichloroacetic acid

The solution was diluted 1:10 in distilled water before use.

**Solutions for fluorescence staining of cells**

<u>0.1M phosphate buffered paraformaldehyde</u>

stock solutions

270 mM sodium dihydrogen orthophosphate

630 mM sodium hydroxide

68 mM calcium chloride

<u>Microtubuli stabilising buffer (tsb)</u>

100 mM PIPES pH 6.9 (Sigma) pH adjusted with 1 N NaOH

0.5% (v/v) triton-100

4% (w/v) PEG 6000

1 mM EGTA

<u>Blocking buffer</u>

1% (w/v) BSA

100 mM glycine

All solutions were made up to the required volume with distilled water, and stored at 4°C.

## *Appendix E - Buffers and solutions*

**Phosphate buffered saline (PBS)**
> 137 mM sodium chloride
> 2.7 mM potassium chloride
> 8 mM di-sodium hydrogen orthophosphate
> 5.3 mM potassium di-hydrogen orthophosphate

PBS was adjusted to pH 7.4 with HCl.

**Potassium phosphate buffer** (Hydroxylapatite column buffer)
> 10 mM or 250 mM potassium di-hydrogen orthophosphate
> 10 mM or 250 mM di-potassium hydrogen orthophosphate

The potassium di-hydrogen orthophosphate was added to the di-potassium hydrogen orthophosphate to give a buffer of pH 7.5.

**Carbonate-bicarbonate buffer** (ELISA buffer)
> 50 mM sodium carbonate
> 50 mM sodium hydrogen carbonate

The sodium carbonate was added to the sodium hydrogen carbonate to give a buffer of pH 9.2.

**Sodium phosphate buffer** (Protein G Sepharose column buffer)
> 20 mM sodium di-hydrogen orthophosphate
> 20 mM di-sodium hydrogen orthophosphate

The sodium di-hydrogen orthophosphate was added to the di-sodium hydrogen orthophosphate to give a buffer of pH 8.

**CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid) transfer buffer**
> stock solution
> > 100mM CAPS (Sigma)

CAPS transfer buffer was adjusted to pH 11 with 2 M NaOH. The stock solution was stored at 4°C.

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