CHAPTER 1

Toxins and the interaction between bacterium and host

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The concept of a bacterial protein toxin was born in the 1880s as Friedrich Loeffler in Berlin, and Émile Roux and Alexandre Yersin in Paris, puzzled over the disease diphtheria. The bacteria were localised in the throats of patients and experimental animals, yet the disease caused systemic damage throughout the body. They reasoned that the bacteria must be producing a poison that could escape from the bacteria to cause widespread damage to the host. So the toxin concept was established right at the start of Medical Microbiology (Roux and Yersin, 1888), only a decade after Robert Koch had established the first definite link between a bacterium and disease with his seminal work on anthrax. However, it was only from the mid-twentieth century onwards that the action of any toxin was understood at the molecular level. Since then progress has been rapid, not only in our appreciation of the mode of action of historically known toxins but also in the discovery of new toxins with novel means of attacking cells.

CLASSES OF BACTERIAL PROTEIN TOXINS

The first toxin to be understood at the molecular level was one from *Clostridium perfringens*, a bacterium notorious for causing wound infections such as gas gangrene. This toxin is a phospholipase that attacks membranes of cells and, thus, it defined one of the three main categories of toxins, i.e., those that attack membranes (MacFarlane and Knight, 1941). The other group of toxins that attacks membranes contains the large number of toxins that insert into membranes to form pores – the pore-forming toxins. It is easy to envisage how these can damage the host cell, although it now transpires that the mode of action of these toxins is more complicated than was first thought.

Toxins of the second major category of toxins act on the cell surface and mimic the action of normal signalling molecules. These toxins are typified by the stable toxin (STa) from *Escherichia coli*. STa is a 19–amino acid peptide that mimics a natural hormone, guanylin, and binds tightly to its receptor in the intestine. This action chronically activates guanylate cyclase activity leading to raised cyclic GMP concentration in the cell and ultimately water exchange into the lumen of the gut and thus diarrhoea (Vaandrager et al., 1992).

Toxins of the third category of toxins enter the cell. The intracellularly acting toxins include such infamous toxins as cholera, diphtheria, and botulinum toxins. All of these attack key targets that are major players in the organisation of the cell and its ability to carry out its normal functions. Each is highly specific and interacts with only one target, or a highly related class of target. Toxins gain entry into the cell in one of two ways. The classical toxins, those that are released from the bacterium, are multidomain proteins that often comprise several subunits. Such toxins have to carry out three unrelated functions (Montecucco et al., 1994). First, they bind to the cell via a receptor to promote uptake into a membrane-bound vesicle. Second, the toxin, or part of it, has to cross the membrane into the cytosol. Finally, the toxin has to interact with the target. For all such toxins identified to date this interaction is an enzymatic one. This is one factor responsible for the extreme potency of these molecules. For example, one molecule of diphtheria toxin is sufficient to kill a cell (Yamaizumi et al., 1978). The other factor that makes these molecules so potent is target selection. Without exception such toxins modify and perturb proteins or processes in the cell that are crucial for its normal function.

Within the last decade or so, a new type of intracellularly acting toxin has been identified. These are generally called effector molecules, although it also seems entirely reasonable to refer to them as toxins. These are delivered directly into the cell by the bacterium, which makes a complex injection machine that forms a selective pore that crosses the two membranes of the bacterium and the membrane of the cell (Cornelis and Van Gijsegem, 2000). There are two slightly different delivery mechanisms that reflect the origin and evolution of these systems; they are referred to as either type III secretion systems (TTSS) or type IV secretion systems (TFSS). Much less is know about type IV secretion systems; in particular, very few of the effectors have been isolated so far (Ding et al., 2003).

Many bacteria inject toxic factors by TTSS, and it is generally the case that each bacterium delivers not one, but a cocktail of several effector proteins into the cell. Each of these toxin effectors has a different but key cellular target.

The discovery of these toxins solved the conundrum of how some of the deadliest bacteria, like *Yersinia*, *Shigella*, and *Salmonella*, were such potent pathogens, although they did not appear to produce classical toxins. Some of the effector toxins are enzymes like the classical intracellular acting toxins, but some mimic normal signalling molecules and bind to signalling molecules to affect their function in a transient manner.

TOXINS THAT MODULATE CELL FUNCTION

The traditional view of a toxin was that of a molecule that caused cellular damage and death at both the cellular and whole-animal level. While this is clearly true for some toxins, such as diphtheria toxin, a picture of toxin action is now emerging in which many toxins act on the signalling mechanisms in the cell in order to take over control of the cell rather than just kill it. This much more subtle tactic enables a pathogen to build a suitable environment for its survival and reproduction. With some bacteria, the preferred environment is intracellular, while other bacteria prefer to avoid phagocytosis.

The background knowledge necessary to understand the mode of action of many of the toxins described in this book has only recently become available, as cell biologists have unravelled the intricate signalling pathways that regulate the cell. Toxin science has greatly aided the advance of cell biology because of the great precision of intracellular acting toxins to pick out a limited set of molecular targets. All the targets chosen by these toxins are important proteins, the majority of which are involved in signalling, while some toxins appear to directly target the cell cycle. The high selectivity and precision of toxin action has in many cases led directly to the identification of signalling proteins and, furthermore, provides a set of valuable reagents for further analysis of these signalling molecules. Indeed, bacterial protein toxins are often referred to as the "cell biologist's toolkit." Toxins such as pertussis toxin and C3 toxin are routinely used by cell biologists to assess the involvement of their targets, the heterotrimeric G-protein G_i and the small G-protein Rho, respectively, in a particular process (Fiorentini et al., 1998; Albert and Robillard, 2002).

It has sometimes been difficult to reconcile the apparently sophisticated action of toxins with their role in disease. The general principle remains that any gene that conveys a competitive advantage to the bacterium will thrive, and it is clear that toxins that affect signalling pathways can aid the colonisation and establishment of the bacteria that express them in a number of ways. By killing cells, they can release a rich source of nutrients for the bacterium. In some cases, the toxin aids dissemination of the bacteria, such

as with cholera. An increasing number of toxins are seen to target cells of the immune system, in particular those involved in innate immunity – the first line of defence against invading pathogens (Guldi-Rontani and Mock, 2002).

While the role of these toxins from the bacterial viewpoint is to aid colonisation of a human or animal host, the perturbation of host cell signalling can lead to various different outcomes at the cellular level that are dependent on both the cell type and signalling pathway affected. For example, toxins that affect proteins of the Rho family can affect the ability of the intoxicated cell to move (Oxford and Theodorescu, 2003). Some toxins can influence differentiation because that process is also controlled by signalling pathways. Similarly, signal transduction that is normally initiated by extracellular regulators that bind to cell surface receptors is intimately involved in the choice between apoptosis and cell growth and division, so it is not surprising that some of the toxins that interfere with signalling can affect that process as well. As a result, some toxins can induce or inhibit apoptosis, and at least one toxin is a potent mitogen.

The cellular signalling system has been honed by evolution over many years. The ability of the bacterium and its toxins to meddle with this finely tuned system that meticulously regulates cell function may carry dangers. Toxins that stimulate growth or apoptosis or inhibit differentiation in many ways behave like tumour promoters or inhibitors of tumour suppression (Lax and Thomas, 2002). This is of particular concern for chronic infections. The possible role of bacteria in carcinogenesis has a long and controversial history that began shortly after the linkage between bacteria and disease was established. There were numerous reports of the appearance of bacteria in tumours from the beginning of the twentieth century onwards. Many of these reports were not published in peer-reviewed journals and they were widely discounted by the mainstream scientific community at the time. Neither the longevity of the carcinogenic process nor the different stages of initiation, promotion, and progression were properly understood at that time, and this hampered a proper assessment of a possible bacterial role in cancer.

The discovery that *Helicobacter pylori* was involved not just in gastric ulceration but also in carcinogenesis forced a re-evaluation of the likely role of bacteria in this process (Parsonnet et al., 1991). It has recently become clear that other bacteria also affect cancer, most notably *Salmonella typhi* in people who become carriers and who, thus, are chronically infected with this bacterium. However, many controversies remain. In particular, the mechanism – or mechanisms – responsible are still being debated. Some scientists view prolonged inflammation caused by chronic infection as being

the main contributing factor to carcinogenesis. However, others suggest more specific and direct effects may be implicated given the close similarities between the action of some toxins and tumour promoters, although there is currently no evidence that they have this role. Parallel work with viruses has shown that some viral infections predispose towards cancer, and the molecular mechanisms here are more clearly understood. Clearly, there is much more to be learned about the role of bacteria in cancer.

The chapters in this book cover not only toxins that explicitly disturb signalling pathways but also bacteria that impinge on cellular function in a similar manner. It is likely that these may well be found to express specific factors that explain these effects. In addition, the likely role of bacteria in the processes of carcinogenesis is discussed.

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CHAPTER 2

The mitogenic *Pasteurella multocida* toxin and cellular signalling

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The *Pasteurella multocida* toxin (PMT) is produced by some type A and D strains of the Gram-negative bacterium *Pasteurella multocida*. These bacteria cause several animal infections and can occasionally cause human disease. PMT is the major virulence factor associated with porcine atrophic rhinitis, a non-fatal respiratory infection characterised by loss of the nasal turbinate bones and a twisting or shortening of the snout. However, PMT is highly toxic to animals, being lethal to mice at similar concentrations to diphtheria toxin. Despite these toxic properties, it turns out that PMT has unexpected effects on cells in culture leading to perturbation of several signalling pathways. The consequence of this action is that PMT can affect the regulation of cell growth and differentiation.

PMT IS A MITOGEN

The cellular effects of PMT have been most widely studied on Swiss 3T3 cells, a mouse fibroblast cell line. These cells are useful for studying growth factors since they are contact-inhibited and are readily quiesced by growing to confluence and allowing the cells to deplete the medium of growth factors. Rozengurt et al. (1990) first showed that PMT caused quiescent Swiss 3T3 cells to recommence DNA synthesis. The toxin is highly potent, inducing maximal DNA synthesis at only 1.25 ng/ml (or about 2 pM). This is equivalent to the DNA synthesis induced by 10% foetal bovine serum. Thus, PMT is more mitogenic for this cell type than any known growth factor (Figure 2.1, top panel). PMT also induces quiesced Swiss 3T3 cells to reinitiate proliferation (Figure 2.1, lower panel). The cells lose their density-dependent growth inhibition in the presence of 10 ng/ml toxin, and more than double in number by 4 days after addition of toxin to confluent monolayers. Addition

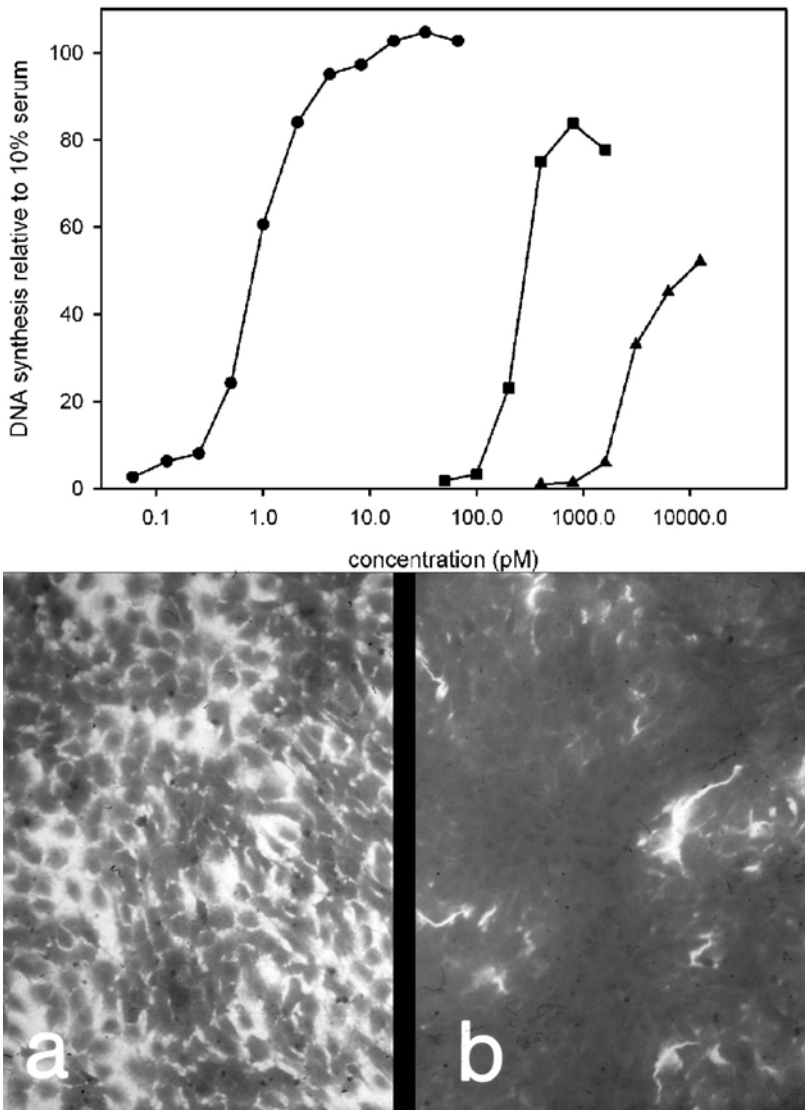

Figure 2.1. PMT is a mitogen for Swiss 3T3 cells. Top panel: relative mitogenicity of PMT (•), platelet derived growth factor (PDGF) (■) and bombesin (▲); lower panel: cell proliferation induced by 48 h PMT treatment: a, untreated cells; b, PMT treated cells. (See color section.)

of PMT to subconfluent cells resulted in a striking proliferation of cells; the final saturation density increased 6-fold after treatment for 11 days. Thus the toxin induces more than one cycle of cell division. Furthermore, when quiescent cells were incubated for 24 h with 10 ng/ml PMT then trypsinised, washed, and replated in the absence of toxin, the subsequent growth of PMT-pretreated cells was markedly enhanced.

PMT is a mitogen for a number of other mesenchymal cells. Several murine cells lines including BALB/c 3T3, NIH 3T3, or 3T6 cells respond to PMT with a striking increase in cell growth. It is also mitogenic for tertiary cultures of mouse embryo cells and human fibroblasts (Rozengurt et al., 1990) and for primary embryonic chick osteoblasts (Mullan and Lax, 1996).

However, PMT does not appear to be a mitogen for certain cell types, such as embryonic bovine lung (EBL) or Vero cells. PMT affects the morphology of these cells, in particular causing cell rounding. This outcome has been regarded as a cytopathic or cytotoxic effect (Rutter and Luther, 1984; Pettit et al., 1993). However, it has been demonstrated that Vero cells treated with toxin remain viable by trypan blue exclusion assay, although they do not undergo DNA synthesis or proliferation (Wilson et al., 2000). The consequences of PMT treatment are therefore dependent on cell type.

PMT ACTS INTRACELLULARLY

There is considerable evidence that PMT is an intracellularly acting toxin. Typically, such toxins bind to a specific cell-surface receptor, and are taken up into cells by receptor-mediated endocytosis. This is followed by penetration through the membrane of the vesicle into the cell cytoplasm where the toxin interacts with a specific target protein. Many bacterial toxins studied to date target and enzymatically modify proteins with key roles in cellular functions such as cell-signalling and growth.

There is a lag period of at least an hour between application of PMT to cells and the onset of a cellular response (Rozengurt et al., 1990). This contrasts with receptor-acting growth factors whose mitogenic effects are seen within minutes. The longer lag is thought to be due to the requirement for toxin internalisation. Methylamine, an agent that increases endosomal and lysosomal pH and thereby inhibits receptor-mediated endocytosis, completely blocked the induction of DNA synthesis by PMT. Similarly, a neutralising antibody specific for PMT blocked the effect of the toxin when added shortly after toxin application, but was ineffective when added at 3 h. Finally, transient exposure of cells to PMT followed by extensive washing and incubation

in toxin-free medium was sufficient to induce DNA synthesis. These experiments demonstrated that PMT acts intracellularly.

Binding and internalisation of PMT has been visualised by labelling purified toxin with colloidal gold (Pettit et al., 1993). Within 1 minute of addition of labelled PMT to Vero or osteosarcoma cells, gold-PMT particles were observed adhering to plasma membranes and in flask-shaped invaginations of the membrane. This binding was to a specific receptor, since it could be competed out by excess unlabelled toxin. After several minutes, the particles could be seen mainly in non-coated pits and in endocytic vesicles. The particles were not seen in vesicles deeper than 500 nm into the cytosol even after several hours. We have used PMT mutated in the catalytic domain in competition assays with wild-type toxin to demonstrate specific saturable binding with a Kd of 1.5 nM (Pullinger et al., 2001). The membrane receptor for PMT has not yet been identified, although binding of gold-labelled PMT was inhibited by mixed gangliosides (Pettit et al., 1993). Similarly, preincubation of PMT with gangliosides GM_1, GM_2 or GM_3 counteracted its effect on DNA synthesis (Dudet et al., 1996), suggesting that the receptor might be a ganglioside.

The translocation of toxins across membranes into the cytosol often involves a low pH processing step. For example, some toxins are proteolytically cleaved in endosomes before the catalytic fragment can be translocated into the cytosol (Olsnes et al., 1993). The finding that PMT action is blocked by the weak base methylamine suggests that PMT may enter the cytosol from an acidic compartment, such as an endosome or lysosome. PMT is highly resistant to proteolysis at neutral pH but becomes susceptible to a number of proteases at pH 5.5 and below, indicating that a conformational change occurs at this pH (Smyth et al., 1995). This was supported experimentally by transverse urea gradient gels and analysis of the circular dichroism profiles, each of which showed a transition in PMT structure at about this pH (Smyth et al., 1999). It is not yet known if PMT is cleaved by proteases *in vivo*.

PMT AFFECTS SEVERAL SIGNALLING PATHWAYS

PMT modifies cellular function by activating a number of cell signalling pathways. Intracellular toxins such as PMT act by subverting these signalling processes. Typically, they physically interact with and modify a specific signalling protein or group of related proteins and either activate or inactivate them. The effects are often long-lasting since the active components of toxins are enzymes. This contrasts with the normal transient activation of cell signalling pathways by receptor-acting growth factors. Thus, the cellular outcome of toxin action may be unusual.