Pathogénie moléculaire et cellulaire des infections bactériennes

Leçon 3

Développement de la maladie : toxines bactériennes et leurs interactions avec les cellules de l'hôte

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Molecular and cellular pathogenesis of bacterial infections

Lecture 3

Development of disease : bacterial toxins and their interaction with host cells

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RESUME : L'étape ultime dans les infections bactériennes est la production d'un effet délétère sur l'hôte avec le développement de lésions cellulaires et tissulaires provoquant des troubles de fonctionnement de divers organes et des signes cliniques. L'effet délétère peut être la conséquence de la production par la bactérie de protéines agissant en perturbant spécifiquement la structure ou le métabolisme des cellules de l'hôte. La première description d'une telle protéine date de 1888, quand deux chercheurs français, Emile Roux et Alexandre Yersin identifièrent la « toxine » diphtérique. Le mot « toxine » utilisé par Roux et Yersin trouve son origine dans le mot grec « *toxicon* » (τοξικον), qui signifie « poison ». Durant la fin du 19e siècle les toxines tétanique et botulique furent décrites par K. Faber en 1889 et par E. van Ermengem en 1898. Et bien d'autres toxines bactériennes furent décrites pendant le 20e siècle.

Aujourd'hui, ces toxines bactériennes sont souvent appelées « exotoxines », même si toutes ne sont pas sécrétées dans le milieu extérieur par la bactérie, pour les différencier des endotoxines, qui représentent des molécules de nature non protéique attachées à la cellule bactérienne, comme les lipopolysaccharides (LPS) des bactéries Gram négatives et les acides teichoïques des bactéries Gram positives. Ces différentes (exo)toxines bactériennes sont aujourd'hui classées selon leur structure et leur méchanismes moléculaires d'action sur les cellules de l'hôte.

Diverses toxines sont ainsi appelées « cytolysines », car elles provoquent la lyse des cellules cibles en agissant sur les membranes cytoplasmiques des cellules cibles. Elles comprennent des phospholipases qui hydrolysent les glycéro-phospholipides et les sphingolipides de ces membranes cytoplasmiques, et trois groupes de toxines formant des pores dans l'épaisseur des membranes cytoplasmiques : celles proches de l'aérolysine d'*Aeromonas hydrophila*, celles dites dépendantes du cholestérol avec la listériolysine de *Listeria monocytogenes* et la famille phylogénétique des toxines RTX *(Repeats in ToXin)* dont le représentant type est l'hémolysine α d'*Escherichia coli*.

De nombreuses autres toxines se caractérisent par une structure de type dimérique et un activité intracellulaire. Elles sont formées de l'assemblage d'une copie d'une sous-unité A et d'une à plusieurs copies d'une sous-unité B, ou d'une seule protéine subdivisée en une domaine A et un domaine B. Les sous-unités/domaines B sont responsables de l'attachement de la toxine à la cellule cible sur un récepteur spécifique, tandis que les sous-unités/domaines A sont porteurs de l'activité toxique de la protéine. Après internalisation par endocytose et passage dans le cytoplasme, les sous-unités/

domaines A interagissent avec leurs molécules cibles de diverses manières (ADP-ribosylation ou autre modification accroissant ou réduisant leur activité ; hydrolyse d'ARN ribosomal ou d'ADN ; inhibition de la sécrétion d'un neuromédiateur…) perturbant le métabolisme et diverses fonctions cellulaires. Certaines toxines possèdent une activité enzymatique intrinsèque.

Les toxines d'un troisième groupe sont des oligopeptides qui agissent par activation, après liaison sur un récepteur transmembranaire spécifique, d'enzymes intracytoplasmiques des cellules cibles provoquant l'initiation d'une cascade d'événements intra-cellulaires et perturbant aussi le métabolisme et diverses fonctions cellulaires.

Les gènes qui codent pour les toxines bactériennes peuvent être d'origine étrangère (en ce incluant une origine eucaryote) et peuvent être localisés sur des bactériophages, des plasmides, des transposons et des îlots de pathogénicité, toutes structures qui seront décrites pendant la 3e leçon. L'expression de nombre de ces gènes est régulée par les conditions de croissance des bactéries (paramètres physico-chimiques du milieu extérieur, température, tension en O_2…).

Les toxines bactériennes ont aussi été utilisées par l'homme avec des buts très différents. Déjà à la fin du 19e siècle, elles furent utilisées, sous forme d'anatoxines, comme composants de certains vaccins pour prévenir des maladies comme la diphtérie, le tétanos et le botulisme. Une des toxines botuliques est aussi utilisée comme agent thérapeutique. Malheureusement, l'homme a aussi eu recours aux toxines bactériennes dans le cadre de la guerre ou du terrorisme bactériologiques (toxines charbonneuse et botuliques). Leur futur réside plus que probablement dans l'utilisation de dérivés non toxiques des cytolysines comme porteur d'épitopes pour la production d'anticorps contre divers facteurs de virulence ainsi que des toxines à activité intra-cellulaire pour délivrer des médicaments anti-cancéreux à l'intérieur même des cellules eucaryotes. D'autre part, les sous-unités/domaines B des toxines dimériques peuvent aussi servir de transporteur à des anticorps monoclonaux produits contre, par exemple, un antigène de surface marqueur d'une lignée de cellules cancéreuses.

INTRODUCTION

Definition of a bacterial pathogen

Let us begin with the definition of a pathogen bacterium proposed by Stanley Falkow in 1997 : « I define a pathogen as being any microorganism whose survival is dependent upon its capacity to replicate and persist on or within another species by actively breaching or destroying a cellular or humoral barrier that ordinarily restricts or inhibits other microorganisms. This capacity to reach a unique host niche free from microbial competition and possibly safe from host defence mechanisms sets the foundation for the expression of specific determinants that permit such microbes to establish themselves within a host and to be transmitted to new susceptible hosts. »

Therefore, to understand the mechanisms of occurrence of organ and tissue lesions during the course of infectious diseases it is « only » necessary to identify the specific determinants that are the basis of the four possible stages of the development of a bacterial disease:

(i) colonisation of the epithelia ;

(ii) crossing of the epithelia to penetrate into the mucosa and submucosa ;

(iii) invasion of the host via the blood stream ;

(iv) production of a deleterious effect on the host cells and tissues.

During the previous lecture the «specific determinants that permit such microbes to establish themselves within a host» by colonising epithelia and mucosae were described. «Specific determinants» that exert a deleterious effect on the host and quite often «permit such microbes to be transmitted to new susceptible hosts» are the subject of this third lecture. Very often the deleterious effects on the host are a direct or indirect consequence of the interaction between a bacterial effector, or toxin, and the host cell functions or architecture.

Definition of a bacterial toxin

The first description of the production by a bacterium of a molecule causing damage to the eukaryotic cells dates from 1888 when Emile Roux and Alexander Yersin identified the diphtheria «toxin». The word «toxin» used by Roux and Yersin probably derives from the Greek word «*toxicon*» (τοξικον), meaning «poison». During the 19th century, the tetanus and botulism toxins were also described by Faber in 1890 and by van Ermengen in 1898. And many more were described during the 20th century.

The bacterial toxins have been named at the time of their identification according to numerous and various criteria : clinical sign or lesion (dermonecrotic toxin, tetanus toxin, any lethal toxin), target organ or tissue (enterotoxin, pneumotoxin), names of bacterial genders or species (streptolysin, perfringolysin, listeriolysin) or of target cells *in vitro* or *in vivo* (Verocytotoxins, leucotoxin, haemolysin) or of pioneers in biomedical sciences (Shiga toxins), a circumstance or an event related to the disease (botulinum toxin, meaning sausage), physico-chemical properties (heat-stable or heat-labile), or when everything else fails a Greek or Latin letter (toxins α, β, ε, ι, exotoxin A), and many other criteria. It is therefore common for the same toxin to carry two or three different names. Today, bacterial toxins are quite often classified according to their structure and molecular mechanism of action.

Five classes of bacterial toxin can therefore be described : the class of cytolysins perturbing the cell cytoplasmic membrane with the release of ions and metabolites and entry of water causing swelling, death and lysis ; the class of A-B toxins interfering with

intracellular metabolism after entering the cytoplasm by receptor-mediated endocytosis ; the class of oligopeptides also interfering with intracellular metabolism, but indirectly by initiating a cascade after binding to the cytoplasmic membrane of the target cells. The last two classes of bacterial toxins are the type III- and type IV-translocated bacterial effectors with effects on the cytoskeleton and the immunotoxins that interact with the cells of the immune system, causing deregulation of the innate or the acquired immune systems. Only the first three classes will be described during this third lecture.

CYTOLYSINS

The contribution of cytolysins to virulence is not always straightforward : killing of cells of the immune defences, release of nutriments, or damage to the epithelium in order to invade deeper tissues and/or adhere to extracellular matrix components are all possibilities. The role of some cytolysins might actually be more to lyse the membrane of the phagosome so that the bacteria can escape the lysosome destruction mechanisms and reach the cytoplasm, than to lyse the cytoplasmic membrane of the cell. The cytolysins themselves include the phospholipases, which hydrolyse the glycerol-phospholipids or sphingolipids of the cytoplasmic membrane, and the three groups of pore-forming toxins (PFT) that create channels inside the cytoplasmic membrane of the eukaryotic target cells, through which ions and metabolites escape while water enters causing swelling, death and lysis. The three groups of PFT are : the aerolysin-like PFTs, the cholesterol dependent PFTs and the RTX family of PFTs.

Phospholipases

The phospholipase bacterial toxins are active on many different cell types and most of them are detected by their *in vitro* haemolytic after growth on blood agar. Nevertheless a few bacterial phospholipases are non-toxic and non-haemolytic enzymes. Many phospholipases can also be detected on egg yolk agar by their lecithinase activity. Bacterial phospholipases have phospholipase C (PLC) activity with different substrate specificity : phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol and/or sphingomyelin. An actual role *in vivo* is proven for only some of them.

Examples of phospholipases C with a proven role *in vivo* are :

- the α toxin of *Clostridium perfringens* causing gas gangrene ;
- the β toxin of *C. haemolyticum* causing bacillary haemoglobinurea and of *C. novyi* causing the black disease, a necrotic hepatitis in ruminants ;
- the dermonecrotic toxin of *Corynebacterium pseudotuberculosis* responsible for epizootic lymphangitis in horses and for caseous lymphadenitis in small ruminants ;
- the phosphatidylinositol-specific and the non-specific PLC of *Listeria monocytogenes*. The former is involved in the escape from the primary phagocytic vesicle and the latter is involved in the escape from the double-membrane vacuole in which the bacteria find themselves during cell-to-cell spread.

The phospholipase C of *C. perfringens* or α toxin was the first bacterial toxin shown in the 1940s to carry an enzymatic activity. This 370 amino-acid protein is a zinc-dependent Ca^{2+}-activated enzyme, active against quite a range of phospholipids, in particular those present in the outer layer of the cytoplasmic membrane of eukaryotic cells, such as phosphatidylcholine and sphingomyelin. The α toxin of *C. perfringens* consists of two structural and functional domains. The N-terminal domain binds the Zn ions. The C-terminal domain (amino-acids 251-370) binds two calcium ions and subsequently exposes at its surface some hydrophobic amino-acids that might interact with the hydrophobic tail of membrane phospholipids. Interestingly the C-terminal domains of weakly toxic and non-toxic phospholipases C lack the specific characteristics of the C-terminal domain of the α toxin. The *C. perfringens* α toxin is clearly involved in the production of gas gangrene. Mutants deficient in its production have a partially or totally reduced pathogenic capacity.

Aerolysin-like PFTs.

The aerolysin of *Aeromonas hydrophila* forms, with the *Staphylococcus aureus* α haemolysin, the α toxin of *Clostridium septicum* and other related toxins, a family of 30 to 50 kDa phylogenetically related PFTs of Gram positive and Gram negative bacteria. The α haemolysin of *Staphylococcus aureus* will serve as an example for the description of the family.

This α haemolysin is a 293 amino-acid-long, 33 kDa protein with three domains : the protein core, the pre-stern domain and the amino-latch domain. In the monomeric structure, the hydrophobic residues of the pre-stern domain are positioned against the protein core. After initial binding to a still unidentified receptor, the toxin monomers diffuse in the external layer of the cytoplasmic membrane and assemble to form ring-like heptameric oligomers.

During the assembly process, the monomers undergo conformational changes, during which the pre-stern domain will completely unfold away from the rest of the molecule into a curved β hairpin, which inserts itself into the width of the cytoplasmic membrane. The seven β hairpins of the heptameric ring form a β barrel, like the bacterial porins of the outer membrane of the Gram negative bacteria. But unlike porins, where all β hairpins belong to one unique molecule, the 7 β hairpins of the β barrel in this case belong to the seven toxins forming the ring-like structure. The conformational change does not require the assistance of any other proteins and all necessary information is contained in the primary structure of the toxin.

In parallel, the amino-latch domain folds out and interacts with the neighbour monomer within the heptameric ring. The final unfolded transmembrane heptameric ring has a mushroom-like structure of around 10 nm in height with a central pore of at least 1 nm, corresponding to the surface of 30 lipid molecules of each layer of the cytoplasmic membrane. The pores are selective only by their size. Cytoplasmic components leave the cell, water enters and the cell dies and lyses, forming, for example, a zone of complete haemolysis after growth on blood agar. This might be a way for these bacteria to reach sources of useful nutriments. But some of these toxins have also been shown to trigger apoptosis in leucocytes and/or to acti-

vate the transcription of genes coding for pro-inflammatory mediators. Clearly, their actual role *in vivo*, which may differ depending on the target cells, still awaits full confirmation.

Cholesterol-dependent PFTs

The second class of pore-forming toxins, or « cholesterol-dependent toxins », have been so named because their initial interaction is probably with the cholesterol present in the membrane of the eukaryotic cells. Their second name of « O_2-labile haemolysins » originates from the loss of haemolytic activity in the presence of oxygen, caused by the oxidation of some cysteine residues. Restoration of activity is possible by adding reducing agents, such as thiols, hence their third name of « thiol-activated toxins ». More than 20 members of the family have been described, with the streptolysin O of *Streptococcus pyogenes* and other β haemolytic streptococci, the perfringolysin O of *Clostridium perfringens*, the listeriolysin O of *Listeria monocytogenes* and the pneumolysin O of *Streptococcus pneumoniae* being the most studied and best understood.

The cholesterol-dependent PFTs have a general mechanism of activity and pore-formation very similar to the aerolysin-like toxins, but with the following main differences. The pore is a much larger barrel with a width of up to 35 nm (versus 1), corresponding to the surface occupied by 1600 lipid molecules (versus 30) in each layer of the cytoplasmic membrane. The width of the pore is due to two characteristics of these PFTs : the presence of several dozens of monomers (up to 50 in some pores), instead of only seven, and the presence of two β hairpins per monomer, instead of one. Their actual role *in vivo* has been elucidated for only a very few, such as the listeriolysin of *Listeria monocytogenes*, responsible in synergy with one of the phospholipases C, for the lysis of the phagocytic vesicle.

RTX PFTs

The RTX toxins form a family of 100 to 200 kDa phylogenetically related proteins of Gram negative bacteria, including some members that are non-toxic enzymes. The presence of several nonapeptide repeats near the COOH-terminus that bind Ca^{++} and are essential for full toxicity is the common structural feature of the RTX toxins and the origin of their acronym after « Repeats in ToXins ». The identity and the number of the repeats differ between RTX toxins. Some members of the RTX family are : the leucotoxins/haemolysins of *Actinobacillus pleuropneumoniae* (Apx) and of *Moraxella bovis* ; the α haemolysin (Hly) and the enterohaemolysin (Ehx) of *Escherichia coli*, the haemolysin of *Morganella morganii* (Mmx) ; the leucotoxins of *Mannheimia haemolytica* (Lkt) and of *Actinobacillus actinomycetemcomitans* (Ltx). A very specific RTX toxin is the bifunctional CyaA toxin of *Bordetella* sp., with both haemolytic pore-forming activity and cytoplasmic adenylate cyclase activity. CyaA is most probably the result of a recombination event between genes coding for a bacterial RTX toxin and for a eukaryotic adenylate cyclase, giving birth to an A-B-like toxin (see next section).

The first RTX toxin was described by Kayser in 1903 and is the α haemolysin of *E. coli* (or Hly). This will serve as a prototype for a more detailed description. The α haemolysin molecule (HlyA) of *E. coli* is synthesised as an immature and inactive protoxin of a molecular weight of 110 kDa. Posttranslational modification performed by HlyC, a strictly specific acyltransferase, activates the proHlyA by the addition of two acyl chains. Correct acylation of all RTX toxins is required to obtain full activity. HlyA, as with all RTX toxins, is secreted by a type I secretion system involving the participation of three other proteins. HlyB is a membrane-bound ATPase providing energy for the secretion of HlyA. HlyD forms a channel through the bacterial membranes with the help of the TolC outer membrane protein. The HlyA, HlyB, HlyC and HlyD-encoding genes form one operon on the bacterial chromosome or on a plasmid.

The 1024 amino-acid-long HlyA protein is functionally subdivided into three domains : the fatty acyl groups, the hydrophobic region and the Ca^{++}-binding repeats. The acyl groups may function as anchoring points to the target cell membrane by increasing the hydrophobicity of the protein and/or are involved in pore formation in target cell membranes. The hydrophobic region represents a transmembrane domain. The Ca-binding domain comprises 13 repeats of the specific nanopeptide sequence.

The receptor of the HlyA has not yet been firmly identified, but the receptors of the LtxA and LktA toxins produced by *A. actinomycetemcomitans* and *M. haemolytica* are subunits of the β integrin antigens found on circulating leucocytes. The binding of HlyA to calcium induces conformational changes and insertion of the hydrophobic region into the cytoplasmic membrane of the eukaryotic cell, leading to the formation of the pore. Experimental results obtained in mice and rats confirm that HlyA participates in the virulence of extraintestinal *E. coli* strains, but its exact role is still uncertain : lysis of erythrocytes or leucocytes, escape from a phagocytic vesicle, sub-haemolytic dose effect on different cell types or stimulation of the innate inflammatory response with overproduction of cytokines are all possibilities. The RTX toxins certainly merit further research in the future.

A-B INTRACELLULAR TOXINS

A-B dimeric bacterial toxins are multifunctional since they must accomplish multiple tasks in order to be effective : binding to the host cell receptor, being internalised, crossing the lipid bilayer of the membrane of the endosomes and performing their intracellular toxic activity. The B subunit is responsible for the binding of the toxin to the eukaryotic host cells, while the A subunit carries the toxic enzymatic activity of the molecule. After binding, the whole complex is internalised by different receptor-mediated endocytosis mechanisms and the A subunit crosses the endocytic vesicle membrane following different mechanisms. Many A subunits are subsequently activated by cleavage inside the cytoplasm into the actual active toxin, the large A1 fragment, and a small fragment, the A2 peptide. The activity of the A subunit interferes with cytoskeleton integrity, with protein synthesis, with DNA metabolism, with secretion pathways or with other enzymatic metabolic activities.

General structure

Some dimeric A-B toxins are composed of one actual A subunit and of 4 to 8 actual B subunits linked by

electric forces. Examples are the cholera, *E. coli* heat-labile, Shiga and pertussis toxins. The subunit-encoding genes form one operon with differential regulation mechanisms to allow synthesis of more B subunits. In other dimeric A-B toxins, such as the C2 toxin of *C. botulinum*, the A and B subunits are not linked in solution via either covalent or non-covalent bonds, nor are their encoding genes on the chromosome. Still other A-B toxins, such as the *Pseudomonas* exotoxin A, the diphtheria toxin, the botulinum and tetanus neurotoxins, the dermonecrotic toxins of *Bordetella* sp. and of *Pasteurella multocida* serotype D, and the cytotoxic necrotising factors of *E. coli*, are actually single proteins with two functional domains corresponding to the A and B subunits. In some of them, the two domains can be separated by bacterial proteases into two actual subunits that remain attached by disulfide bonds. Finally, a few A-B toxins are trimeric toxins composed of one A and one B subunit, and of another protein whose role is to work synergistically with the B subunit, as in the cytolethal distending toxins, or to perform another toxic activity, as in the anthrax toxin, with one B subunit and two different A subunits. The encoding gene arrangements vary from one operon to independent genes.

Entry into the cell cytoplasm

After attachment to the target cell cytoplasmic membrane, most A-B toxins are internalised by receptor-mediated endocytosis. A very small number of toxins, like the CyaA of *Bordetella* sp are internalised by direct translocation of the catalytic A subunit or domain. But the CyaA toxin is a particular bifunctional toxin with both haemolytic (pore-forming) and invasive (enzymatic) properties (see previous section). The pore-forming moiety actually permits direct passage through the cytoplasmic membrane into the cytoplasm of the enzymatic active moiety.

i) Receptor-mediated endocytosis

Irrespective of the toxin structure, several mechanisms account for the internalisation of toxins by receptor-mediated endocytosis : clathrin-coated pits as for a number of hormones and growth factors and for the diphtheria toxin, the *Pseudomonas* exotoxin A, the Shiga toxins and the cholera toxin ; cholesterol rich membrane invaginations, or caveolae, for the cholera toxin in particular ; other less characterised clathrin and caveolae independent receptor-mediated mechanisms for the cytotoxic necrotising factors of *E. coli* and partly for the cholera and Shiga toxins. Moreover different mechanisms can account for the entry of the same toxin into different cells. For example, the cholera toxin uses preferentially the caveolae mechanism in enterocytes, but only clathrin-dependent endocytosis to enter neurone cells and even other mechanisms in other cells.

ii) Migration toward the Golgi apparatus

After internalisation, a number of dimeric toxins with several copies of the B subunits migrate in a retrograde manner toward the Golgi apparatus, following different pathways not yet fully understood and are transported into the endoplasmic reticulum. Then the toxins use the Sec61 transmembrane protein complex of the cell sorting mechanism to translocate a partially unfolded A subunit across the endoplasmic reticulum membrane into the cytoplasm. These bacterial toxins are thus exploiting the endoplasmic reticulum translocation machinery to gain access to the cytoplasm. In the cytoplasm, the A subunit is cleaved at an internal site by a host protease to generate the activated catalytic A1 fragment. Archetypes are the cholera toxin, the heat-labile enterotoxins of *Escherichia coli*, the Shiga toxins and the pertussis toxin.

iii) Translocation mechanisms

Other A-B dimeric toxins become « translocation competent » upon acidification of the endosome, via conformational changes. A first translocation mechanism is followed by monomeric toxins that possess a hydrophobic translocation (T) subdomain within the B domain. The conformational changes at low pH within the endocytic vesicle lead to exposure of hydrophobic regions of the T sub-domain that create a membrane channel through which the A domain reaches the cytoplasm. The A domain is subsequently released and activated by cleavage of a peptide bond or of a disulfide bridge. Examples are the exotoxin A of *Pseusomonas aeruginosa*, the diphtheria toxin and the botulinum and tetanus neurotoxins.

A second translocation mechanism from the acidified endosome is followed by dimeric and multimeric toxins produced particularly by the *Bacillus* and *Clostridium* species, with only one copy of a B subunit and whose A-B subunits are not linked to each other before reaching the host cell surface. This is the case, for example, for the dimeric C2 toxin of *C. botulinum* and the trimeric anthrax toxin. The B+T subunits of these toxins bind to their carbohydrate receptors at the cell surface and subsequently expose a specific high-affinity site to which the respective A subunits bind in turn. In the acidified endosome, the B subunit undergoes a conformational change and forms a heptameric ring in the endosome membrane. The A subunit translocates into the cytoplasm of the eukaryotic cell through the heptameric ring channel. These B+T subunits thus resemble very much the aerolysin-like PFTs. After entering the cytoplasm the A subunit is cleaved at an internal site by a host protease to generate the activated catalytic A1 fragment.

Enzymatic activity

The activated A subunits interact with their molecular targets by ADP-ribosylation, glucosylation, deamidation, glycosidation increasing or inhibiting their activity, or by hydrolysis abolishing their activity. Other A subunits actually possess intrinsic enzymatic activity. The changes in cellular activity are the basis for the development of cell, tissue and organ lesions and of clinical signs.

i) ADP-ribosylation of the targets

- different dimeric toxins with one A subunit and 4 to 8 subunits, such as the cholera toxin (CT), the *E. coli* heat-labile enterotoxins (LT1 and LT2) and the pertussis toxin of *Bordetella pertussis*, hyperactivate the G family of GTP-binding proteins. These proteins, among other functions, regulate the activity of the adenylate cyclase, and cause hypersecretion of water and electrolytes with occurrence of watery diarrhea in cholera and enterotoxigenic *E. coli* infections and of paroxysmal cough in whooping cough ;
- several bacillus and clostridial toxins, with independent A-B subunits,

such as the ι toxin of *C. perfringens*, the ι-like toxin of *C. spiroforme*, the C2 toxin of *C. botulinum* and the vegetative insecticidal peptide (VIP) of *Bacillus cereus*, inhibit the formation of polymerised actin-F filaments by ADP-ribosylation of the monomers of γ actin (G actin). This causes perturbation of the morphologic cell modifications that are important in leucocyte migration, endocytosis, and maintenance of tight junctions enhancing epithelium permeability, resulting in fluid accumulation in the intestinal tract and diarrhea, as in the case of *C. spiroforme* infection in rabbits ;

- the monomeric diphtheria toxin and the exotoxin A of *Pseudomonas aeruginosa* block protein synthesis by ADP-ribosylation of the elongation factor 2 and cause cell death, tissue necrosis and development of necrotic pseudomembranes, for example, in the pharynx in the case of diphtheria.

ii) Other modifications of the target molecules

- several large monomeric clostridial toxins, ToxA and ToxB of *C. difficile*, haemorrhagic toxin (HT) and lethal toxin (LT) of *C. sordellii* and α toxin of *C. novyi*, inhibit the activity of the Rho family of GTP-binding proteins by glucosylation. This causes perturbation of the cell morphology and tissue necrosis in the subcutaneous tissue, the liver or the intestine, as in the pseudomembraneous colitis caused by *C. difficile* ;
- the monomeric dermonecrotic toxins of *Pasteurella multocida* and of *Bordetella bronchiseptica* responsible for atrophic rhinitis and the cytotoxic necrotising factors (CNF) of *E. coli*, hyperactivate the Rho family of GTP-binding proteins by deamidation. They cause cytoskeleton rearrangements and initiation of DNA replication with occurrence of stress fibres and of multinucleated cells, leading to cell death and necrosis, as after intradermal injection of CNF, or as in porcine atrophic rhinitis at the level of the turbinates.

iii) Destruction of the target molecules

- the dimeric Shiga toxins (Stx) of *Shigella dysenteriae* and of *E. coli*, with one A and five B subunits, have N-glycosidase activity and cleave a purine residue from the 28S rRNA, altering the function of the ribosomes. These are no longer able to interact with elongation factors EF1 and EF2 and therefore inhibit protein synthesis within the target cells, mainly the endothelial cells of the small arteries, causing fluid leakage, as in the oedema disease in piglets, or internal organ failure, for example at the height of the kidneys in the haemolytic uraemic syndrome in humans ;
- the monomeric tetanus toxin of *Clostridium tetani* is a zinc endoprotease that blocks the release of the GABA (γ aminobutyric acid) regulator of the neurotransmission by hydrolysis of the SNARE (SyNaptosome-Associated protein <SNAP> REceptors) proteins at the height of inhibitory nerve endings, causing spastic paralysis ;
- the monomeric botulinum toxins of *Clostridium botulinum* are other zinc endoproteases that block the release of the acetylcholin neurotransmitter by hydrolysis of the SNARE proteins at the level of the neuromuscular junction, causing flaccid paralysis. The tetanus and botulism toxins are described in more detail in the next section ;
- the trimeric anthrax toxin of *Bacillus anthracis* comprises one B subunit, the protective antigen (PA) and two A subunits with different activities : the Lethal factor (LF) is a zinc endoprotease cleaving the mitogen-activated protein kinase kinases (MAPKK) family causing different cell perturbations and possibly macrophages to release cytokines, leading to shock and death ;
- the trimeric cytolethal distending toxins (CDTs) produced by several bacterial species of the genders *Escherichia*, *Campylobacter*, *Haemophilus*…, cause or mimic DNA damages. They cause accumulation in a phosphorylated inactive form of a cyclin-dependent kinase, blocking the cell division at the G2/M stage, causing the occurrence of giant mononucleated cells with twice the DNA content of a normal cell and leading to cell death by apoptosis.

iv) Toxins with intrinsic activity

The second A subunit of the anthrax toxin of *Bacillus anthracis*, or Oedema toxin (EF), and the CyaA toxin of *Bordetella* sp. are adenylate cyclases. These cause cAMP-induced disruption of many cell functions, including control of the flow of ions and water, with production of oedema in subcutaneous anthrax or of hypersecretion in the respiratory tract in whooping cough, respectively.

These different A-B intracellular toxins thus share similar traits in their general structures and interaction with the target cells, but differ in their precise mechanisms of toxicity and target molecules. It can also be said that more and more toxins acting intracellularly with previously unknown structure and molecular mechanism of action actually belong to one or other group of this huge class of A-B intracellular toxins, as determined after identification of their encoding genes.

Transcytosis

Most A-B intracellular toxins act on the site of infection. A few, however, travel much further than the producing bacteria. They cross the epithelial layer by transcytosis and are carried by the blood stream to internal tissues and organs, sometimes throughout the whole body. Examples are the tetanus and botulism neurotoxins and the Shiga toxins. The mechanisms by which they cross the epithelial barriers and tissues are being progressively uncovered. However, the reasons are still unknown as to why some toxins of the same family are delivered from the endoplasmic reticulum into the cell cytoplasm, while others can accomplish transcytosis and, further, why in some cells, one toxin accomplishes transcytosis, while in other cells the same protein is toxic.

In vivo, the Shiga toxins, for example, are produced in the intestines after colonisation by the verotoxigenic *E. coli* strains, then cross the intestinal wall and enter the blood stream, causing toxaemia, before reaching their cellular targets, the endothelial cells of the small arteries. They cross the intestinal epithelium by transcytosis, being most likely delivered from the Golgi apparatus to the cytoplasmic membrane of the basal pole of the enterocyte.

OLIGOPEPTIDE TOXINS

The oligopeptide toxins interact with the target host cells by fixation on a

specific receptor of the cytoplasmic membrane. This fixation event activates an intracellular component linked to the receptor which in turn activates an intracellular cascade of enzymatic events, leading to perturbation of the cellular metabolism and to hypersecretion.

Oligopeptide toxins range in size from 17 to 48 amino-acids and are reminescent of peptide hormones of multicellular organisms. The archetypes of oligopeptide toxins are the heat-stable enterotoxins (STas) of enterotoxigenic *Escherichia coli*. Enteroaggregative *E. coli* and several other bacterial species produce similar STa toxins : non-O1 *Vibrio cholerae*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Enterobacter aerogenes*… STas of enterotoxigenic *E. coli* are synthesised as preprotoxins of 72 amino-acids and are secreted by the general Sec secretion pathway. After removal of the 18 amino-acid long signal sequence, further processing of the protoxin occurs in the periplasm and/or after the passage through the outer membrane of the bacterial cell to deliver an 18 amino-acid toxic peptide of less than 2kDa.

The minimal active peptide of the STas of *E. coli* comprises 13 amino-acids, from amino-acids 5 to 17, with three disulfide bridges : between cysteines 5 and 10, 6 and 14, and 9 and 17. The identity and 3D structure of these 13 amino-acids are primordial for the full activity of the toxin and any substitution induces a change of activity. The correctness of the disulfide bonds is also necessary for full biological activity : mutation of residues 6 or 17 fully abolishes toxicity and mutation of residues 5 or 10 reduces it. The STas of *E. coli* bind to the brush border membrane of enterocytes. The receptor is the transmembrane protein with guanylate cyclase activity, which is present in the apical pole of the intestinal cells. The guanylate cyclase is responsible for regulating intracellular levels of cyclic guanosine monophosphate (cGMP). cGMP is an important signalling molecule in eukaryotic cells, and changes in its level affect a number of cellular processes, including activities of ion pumps. The electrolyte flux in the bowel is affected, with inhibition of sodium absorption and stimulation of chloride and water secretion, leading to watery diarrhoea. The guanylate cyclase also acts as the physiological receptor of a eukaryotic peptide hormone, named guanylin. The effect of guanylin is nevertheless not as dramatic as the effect of STas and its function may be to keep the mucus layer wet.

TETANUS AND BOTULISM NEUROTOXINS

Research work at the molecular and genetic levels sometimes reveals unexpected and surprising data about bacterial toxins, like the tetanus toxin (TeTx) and the botulism toxins (BoNT) as belonging to the same family on the basis of similarities in genetics, structure, enzymatic activity and targets inside the cells of the nervous system. This is despite the fact that these two neurotoxins produce opposite clinical signs : the TeTx causes a spastic paralysis, while the BoNTs cause a flaccid paralysis.

The TeTx is unique and is produced by *C. tetani*. In contrast, the BoNTs fall into seven different toxin types according to their antigenic properties and are produced by clostridia belonging not only to the three taxonomic groups of the species *C. botulinum*, but also to other species like *C. baratii*, *C. butyricum* and *C. argentinense*. Very roughly BoNT/A, B, E, and less frequently F and G, are responsible for human botulism, whereas BoNT/C and D are the main causative types involved in animal botulism. After cloning of the respective genes, it was discovered that the TeTx-encoding gene shares similarities with all BoNT-encoding genes in the sequence and general organisation.

The TeTx and BoNTs therefore share the common structure of a monomeric A-B toxin and are produced as single chain proteins of about 150 kDa. These are either inactive or poorly active and are proteolytically activated into a light chain (L) (approximately 50 kDa) and a heavy chain (H) (approximately 100 kDa). Both chains remain linked by a disulfide bridge. The overall structure shows three distinct domains: the enzymatic A domain corresponding to the L chain, the translocation T domain corresponding to the N-terminal part of the H chain and the binding B domain constituted by the two subdomains corresponding to the C-terminal part of the H chain. The L chain also contains the characteristic zinc-binding motif of zinc metalloproteases (His-Glu-Xaa-Xaa-His).

The TeTx and BoNTS also share common mechanisms of action. These different proteins possess a signal peptide and are classically excreted by the general Sec secretion pathway. The toxins then migrate toward their respective target cells. Few data are available to explain the transcytosis of the BoNTS through the intestinal epithelial cells and the migration of the BoNTs and TeTx in the tissue and in the blood stream. The B domains of TeTx and BoNTs then recognise different specific receptors on the terminal nerve endings, but the identity of each receptor is still controversial. The receptors probably consist of a ganglioside associated to a protein. The neurotoxins bound to their receptor are internalised into endocytic vesicles. The vesicles containing BoNTs remain at the nerve extremity, while TeTx vesicles are transported in a retrograde manner along the axon until they reach the cell body in the spinal cord. In the spinal cord, the TeTx is transported across the synaptic cleft into the inhibitory interneurons, by a still unidentified mechanism.

Acidification of the endosomes then triggers conformational change of the neurotoxins, which insert their T domain into the vesicle membrane, forming a channel through which the A enzymatic domains or L chains translocate into the cytosol. The TeTx and BoNTs block the neuroexocytosis machinery responsible for the neurotransmitter release at neuron terminals by proteolysis of the specific SNARE (SNAP proteins REceptors) proteins involved in synaptic vesicle fusion with the presynaptic membrane. BoNT/B, D, F and G and TeTx cut synaptobrevin or VAMP (Vesicle-Associated Membrane Protein) ; BoNT/A and E cut SNAP25 (SyNaptosome-Associated Proteins of 25 kDa) ; BoNT/C cuts both SNAP25 and syntaxin. The cleavage sites are different, except for BoNT/B and TeTx, which cut synaptobrevin at exactly the same site.

Thus, the BoNT/B and TeTx share an identical molecular mechanism, while inducing opposite clinical signs. This clearly indicates that the difference in clinical signs results from the different sites of action. The BoNTs act at the neuromuscular junctions by preventing the regulated release of acetylcholine, leading to flaccid paralysis. The blockade of the liberation of glycine or GABA in the inhibitory interneurons by TeTx disrupts the balance between stimulations from the sensitive nerve

and the motorneuron, leading to a permanent excitation of the muscles with characteristic spastic paralysis.

OF TOXINS AND MEN

If the majority of bacterial toxins are key bacterial effectors in diseases, they have also been used by humans for various purposes. Let us briefly review the usages of bacterial toxins in therapy, biological warfare and immunisation, beginning with their use in therapy.

In therapy

The concept of using microbial toxins for the treatment of physiological disorders in humans arose progressively during the years as a result of many research studies and collaborations between physicians, physiologists, pharmacologists and toxicologists.

i) Botulinum toxins

For example, the botulinum A (BoNT/A) toxin is used in the treatment of permanent muscle contraction. The specific site of action of the BoNT toxins was demonstrated in the 1940s and the concept of its therapeutic use arose progressively during the 1950s/1960s with the first experiments in monkeys. During the 1970s the BoNT/A toxin was successfully utilised for correction of strabismus. In 1979, the US Food and Drug Administration approved the production of toxin batches for the treatment of strabismus, blepharoplasm and hemifacial spasm.

Since these first experimental steps, the BoNT/A toxin has been shown to be effective in humans, not only in the treatment of other muscle problems (involuntary contractions, dystonias, spasticity, writer's cramp and tics), but also in pain syndromes (myofacial pain, tension and migraine headaches). It received approval for use in 1989. Experimentally it is also being tested in animal models for conditions for which muscle atrophy is desired, as in the case of prostate involution or muscle immobilisation.

Early scientists noted that the BoNT/A toxin showed no known focal or systemic effects, did not elicit immune response at the doses used, diffused slowly out of the injected muscle into adjacent muscles, acted for several weeks to months and showed a dose–effect relationship. Since then, some problems have arisen, such as antibody production and diffusion into other muscles. Another problem is the need for repeated injections in the case of chronic disorders, as this treatment is only symptomatic and not aetiological. Scientists consequently tried other BoNT serotypes, but they appear to be less efficient. In the future, use of the whole natural toxin may be replaced by use of conjugated engineered toxin peptide fragments, thanks to rapidly advancing knowledge of the structure/physiology of the botulinum and tetanus neurotoxins.

ii) Toxins as anticancer drugs

The activities of several potent cytotoxins have been harnessed as potential therapies for certain cancers, either directly by their cytotoxicity or as components of immunotoxins. The Shiga toxins, for example, bind to the Gb3, or cell surface glycolipid CD77 receptor, which is expressed by B cells in certain B-lymphosarcomas. The Shiga toxins can purge murine bone marrow of malignant CD77+ B cells before autologous bone marrow transplant.

The *Pseudomonas* exotoxin A can also be engineered as the cell-killing component of imunotoxins, whose second component is a monoclonal antibody. Such hybrid molecules are tested in clinical trials for the treatment of B-cell lymphomas of leukaemia and for bone marrow transplants.

The anti-tumour effects of *C. difficile* A toxin and of *C. perfringens* enterotoxin are also being tested in *in vitro* experiments on cell lines.

In biological warfare and terrorism

Though bacteria have long been used in biological warfare, as explained during the inaugural lecture, use of purified, or semi-purified toxins, is a relatively new topic that developed during the 20th century. This short discussion is limited to *C. botulinum* which has attracted the most attention. One of the first documented uses of botulinum toxin as a weapon occurred in the 1930s in Mongolia by the sinister Japanese Unit 731. Later, during World War II, the USA developed botulinum toxin as a bioweapon. The USA programme with botulinum toxin officially ceased between 1969 and 1970. In the context of the use of botulinum toxin as an agent of bioterrorism, it has been suggested that the toxin could be released either in an airborne form, or used to contaminate food supplies. In Japan, the Aum Shinrikyo cult reportedly isolated *C. botulinum* from soil and used this strain to produce botulinum toxin. Fortunately, several attempts to release aerosols of the toxin in Tokyo between 1990 and 1995 failed.

In immunisation

As for adhesins, the most beneficial and remarkable utilisation of the bacterial toxins is certainly the production of vaccines to develop protection against several bacterial diseases in man and animals. As early as December 1890, Emil von Behring and Shibasaburo Kitasato immunised animals with tetanus toxin preparations and discovered neutralising antitoxin antibodies in the sera of the animals. The development of a vaccine with the diphtheria toxin is the second most well known successful story of the molecular bacteriology during the pre-molecular era.

Typically the toxins are incorporated into vaccines after semi-purification of culture supernatants and detoxification, that is usually performed by formaldehyde and heat treatment, to produce so-called « toxoid vaccines » (or « anatoxins » in French). Toxoids are thus detoxified toxins retaining their immunogenicity. More recently, the genetic engineering has allowed the development of toxoid vaccines following a more systematic approach and some current vaccines contain genetically engineered toxin-derivatives. But only a very few bacterial infections are actually based solely on the production of toxins. There are also adhesins and other surface antigens that help the bacteria to survive inside the host body. Many toxoid vaccines therefore also contain the whole cell bacterium, because experiments have shown additional protective effect of cell surface antigens. These are called bacterin::toxoid vaccines.

The following vaccines, with at least one toxin component specifically referred to, are commercialised in human and veterinary medicine in Belgium :

- different clostridial vaccines against enterotoxaemia, internal infections and tetanus of ruminants and pigs, with exotoxins of *Clostridium perfringens*, *C. noyi*, *C. haemolyticum*, *C. chauvoei*, *C. septicum* and/or *C. tetani* ;
- tetanus vaccines in humans and horses, based on the tetanus toxin of *C. tetani* ;
- diphtheria vaccine in humans, based on the diphtheria toxin of *C. diphteriae* ;

- atrophic rhinitis vaccine in piglets with the different toxins of *Bordetella bronchiseptica* and the dermonecrotic toxin of *Pasteurella multocida* serotype D. One vaccine contains a genetically engineered dermonecrotic toxin of *Pasteurella multocida* with a deletion of the toxic moiety while retaining the immunogenicity ;
- possibly also one vaccine against whooping cough in humans with extracts of *Bordetella pertussis* ;
- vaccines against neonatal diarrhoea in piglets with the heat-labile enterotoxin of porcine enterotoxigenic *Escherichia coli* in addition to the fimbrial and bacterial antigens ;
- vaccines against contagious pleuropneumoniae in piglets with three different PFTs of the RTX family, produced by *Actinobacillus pleuropneumoniae*.

Another area of the use of bacterial toxins in immunology is the carriage of peptides and epitopes inside the eukaryotic cells of the immune system to provoke the development of an immune response against these foreign sequences. It is very interesting to mount an immune response against oligopeptide toxins that are non-immunogenic *per se*, against non-immudominant epitopes of large toxins, against epitopes of minor subunits of fimbrial adhesins and against epitopes provoking a cell-mediated immune response. These artificial proteins, named polytops, open new perspectives in the field of vaccination but are still in the experimental stages.

CONCLUSION

During the second and third lectures, we travelled throughout the worlds of bacterial colonisation factors and toxins, dealing mainly with the interactions between these virulence factors and the host cells. The genetics of several adhesins and toxins and the DNA structures carrying their encoding genes are the subjects of the following lectures, including the regulation mechanisms of their expression.

FURTHER READINGS

BONVENTRE P.F. The nomenclature of microbial toxins : problems and recommendations. In : Ajl S.J., Kadis S., Montie T.C. (Eds), Microbial toxins : a comprehensive treatise. Volume I : bacterial protein toxins. Academic Press : London, 1970, 29-66.

BURNS D.L., BARBIERI J.T., IGLEWSKI B.H., RAPPUOLI R. Bacterial protein toxins. ASM Press : Washington, 2003, 348 p.

DONNENBERG M.S., WELCH R.A. Virulence determinants of uropathogenic *Escherichia coli*. In : Mobley H.L.T., Warren J.W. (Eds), Urinary tract infections : molecular pathogenesis and clinical management. ASM Press : Washington, 1996, 135-174.

FALKOW S. What is a pathogen ? *ASM News*, 1997, **63**, 359-365.

GYLES C.L. *Escherichia coli* enterotoxins. In : Gyles C.L. (Ed), *Escherichia coli* in domestic animals and humans. CAB International : Wallingford, 1994, 337-364.

KAYSER H. Ueber Bakterienshämolyse, im Besonderen das Colilysin. *Zeitschr. Hyg. Infektionkrankh*., 1903, **42**, 118-138.

KRUEGER K.M., BARBIERI J.T. Bacterial ADP-ribosylating exotoxins. In : Roth J.A., Bolin C.A., Brogden K.A., Minion F.C., Wannemuehler M.J. (Eds), Virulence mechanisms of bacterial pathogens. 2nd Edition. ASM Press : Washington, 1995, 231-242.

LALLI G., BOHNERT S., DEINHARDT K., VERASTEGUI C., SCHIAVO G. The journey of tetanus and botulinum neurotoxins in neurons. *Trends Microbiol.*, 2003, **11**, 431-437.

NATARO J.P., SEARS C., FASANO A., BLOCH R.J. Enteric microbial toxins and the intestinal epithelial cytoskeleton. In : Hecht G.A. (Ed), Microbial pathogenesis and the intestinal epithelial cell. ASM Press : Washington, 2003, 301-332.

POPOFF M. Molecular biology of clostridial neurotoxins. In : Duchesnes C., Mainil J., Popoff M., Titball R. (Eds), Proceedings of the 3rd Workshop of the Concerted Action QLK2-CT-2001-01267 (Protein toxins of the genus *Clostridium* and vaccination, Salisbury, UK, November 2002). Presses de la Faculté de Médecine vétérinaire de l'ULg : Liège, 2003, 25-44.

RAPPUOLI R., PIZZA M. Bacterial toxins. In : Cossart P., Boquet P., Normak S., Rappuoli R. (Eds), Cellular microbiology. ASM Press : Washington, 2000, 193-220.

RODIGHIERO C., LENCER W.I. Trafficking of cholera toxin and related bacterial enterotoxins : pathways and endpoints. In : Hecht G.A. (Ed), Microbial pathogenesis and the intestinal epithelial cell. ASM Press : Washington, 2003, 385-402.

ROUX E., YERSIN A. Contribution à l'étude de la diphtérie. *Ann. Inst. Pasteur*, 1888, **2**, 629-661.

RUTTER J.M. Bacterial toxins as virulence determinants of veterinary pathogens : an overview. In : Roth J.A. (Ed), Virulence mechanisms of bacterial pathogens. ASM Press : Washington, 1988, 1213-1227.

SALYERS A.A., WHITT D.D. Bacterial pathogenesis : a molecular approach. 2nd Edition. ASM Press : Washington, 2002, 539 p.

TITBALL R., MAINIL J., DUCHESNES C., POPOFF M. Protein toxins of the genus *Clostridium* and vaccination. Presses de la Faculté de Médecine vétérinaire de l'ULg : Liège, 2003, 50 p (also available on the website http://www.genusclostridium.net)

TURTON K., CHADDOCK J.A., ACHARYA K.R. Botulinum and tetanus neurotoxins : structure, function and therapeutic utility. *Trends Biochem. Sci.*, 2002, **27**, 552-558.

TWETEN R.K. Pore-forming toxins of Gram-positive bacteria. In : Roth J.A., Bolin C.A., Brogden K.A., Minion F.C., Wannemuehler M.J. (Eds), Virulence mechanisms of bacterial pathogens. 2nd Edition. ASM Press : Washington, 1995, 207-230.

VAN BOST S., MAINIL J. Facteurs de virulence et propriétés spécifiques des souches invasives d'*Escherichia coli* : II) Production de toxines. *Ann. Méd. Vét.*, 2003, **147**, 327-342.

VAN HEYNINGEN W.E. General characteristics. In : Ajl S.J., Kadis S., Montie T.C. (Eds), Microbial toxins : a comprehensive treatise. Volume I : Bacterial protein toxins. Academic Press : London, 1970, 1-28.

WELCH R.A. Phylogenetic analyses of the RTX toxin family. In : Roth J.A., Bolin C.A., Brogden K.A., Minion F.C., Wannemuehler M.J. (Eds), Virulence mechanisms of bacterial pathogens. 2nd Edition. ASM Press : Washington, 1995, 195-206.