Pathogénie moléculaire et cellulaire des infections bactériennes

Leçon 2

Colonisation des muqueuses : facteurs d'adhésion et leurs interactions avec les cellules de l'hôte

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Molecular and cellular pathogenesis of bacterial infections

Lecture 2

Colonisation of the mucosae

Adherence factors and their interaction with host cells

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RESUME : La première étape de nombreuses infections bactériennes est la colonisation des surfaces de l'hôte, c'est-à-dire les muqueuses et la peau. L'adhésion des bactéries aux muqueuses est médiée par des structures présentes à la surface de la cellule bactérienne, appelées adhésines, qui interagissent avec des composant présents à la surface de la cellule eucaryote ou à hauteur de la matrice extra-cellulaire, appelés récepteurs. Les propriétés d'adhésion des bactéries furent décrites pour la première fois au début du 20e siècle, mais l'étude des adhésines bactériennes ne débuta véritablement que dans les années 1950. Aujourd'hui des dizaines d'adhésines bactériennes sont connues, ainsi que trois types d'interactions adhésines-récepteurs : protéine-carbohydrate, protéine-protéine et hydrophobine-hydrophobine.

Les lectines sont les adhésines bactériennes les plus étudiées. De nature protéique, elles ont été classées en différents groupes sur base de leurs propriétés d'hémaglutination, leurs ultrastructures et leurs spécificités de récepteurs. Parmi les bactéries Gram négatives, les lectines du premier groupe sont responsables d'hémaglutination sensible au mannose et présentent une structure de nature fimbriaire (fimbriae de type 1) ; celles du deuxième groupe sont responsables d'hémaglutination résistante au mannose et présentent une structure de nature fimbriaire ou fibrillaire ; celles du troisième groupe sont aussi responsables d'hémaglutination résistante au mannose, mais ne sont ni des fimbriae, ni des fibrillae ; enfin, celles du quatrième groupe ne provoquent pas d'hémaglutination. Les lectines de type fimbriaire ou fibrillaire sont si nombreuses que plusieurs schémas de classification ont été proposés, basés sur leur ultra-structure précise, leur composition moléculaire et/ou leur spécificité antigénique, sans qu'aucune ne donne pleine satisfaction. Les lectines des bactéries Gram positives sont associées au peptidoglycan de ces bactéries et représentent des structures distinctes apparaissant à la surface de la cellule bactérienne. Les récepteurs de nature carbohydrate de ces adhésines de type lectine sont des domaines de glycoprotéines ou glycolipides assurant les contacts entre cellules de l'hôte ou faisant partie d'une cascade intra-cellulaire de transmission d'un signal d'origine externe. Pour certaines lectines cependant, le récepteur est de nature oligo-peptidique et fait partie d'une protéine plus grande.

L'adhésion bactérienne peut aussi s'accomplir par l'intermédiaire de protéines de la surface bactérienne, présentes dans la membrane externe de la bactérie Gram négative ou associée au peptidoglycan de la bactérie Gram positive, qui interagissent avec des protéines présentes dans la membrane cytoplasmique de la celllule eucaryote ou dans la matrice extra-cellulaire. Le troisième type d'adhésion bactérienne aux surfaces de l'hôte est assurée par des composants hydrophobes des surfaces des cellules

bactériennes et eucaryotes (= hydrophobines), le plus fréquemment des lipides et des domaines hydrophobes de protéines.

La colonisation des surfaces de l'hôte par les bactéries est suivie de la production de toxines à action locale ou systémique, après transport dans la circulation sanguine, sur les cellules de l'hôte. L'adhésion peut aussi être suivie de l'injection d'effecteurs bactériens à l'intérieur de la cellule de l'hôte, provoquant des réarrangements à hauteur du cytosquelette et, le plus fréquemment, de l'entrée par phagocytose forcée de la bactérie dans la cellule eucaryote.

Les gènes qui codent pour les adhésines bactériennes peuvent être localisés sur des plasmides ou des îlots de pathogénicité. Leur expression est fréquemment sous l'influence des conditions physico-chimiques extérieures de croissance (composition chimique du milieu, température, concentration en oxygène …) (voir leçon # 4). Les adhésines bactériennes ont aussi été utilisées par l'homme comme valences vaccinales, plus particulièrement contre les maladies entériques. Le futur réside probablement en l'utilisation de diverses adhésines, comme porteur d'épitopes étrangers, afin de produire des anticorps contre différents facteurs de virulence.

INTRODUCTION

Definition of a pathogen bacterium

A definition of a pathogen bacterium was 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 and the mucosae ; (iii) invasion of the host via the blood stream ; (iv) production of a toxic effect on the host cells and tissues. Stages 1 and 4 are achieved by all bacteria responsible for infections of the digestive, respiratory, urinary and genital tracts, of the skin and of the conjunctiva and are the subjects of the present and third lectures.

In order to colonise a mucosal surface a pathogen bacterium must first overcome physical defences, such as the mucus blanket, peristalsis, fluid flow, beating cilia, antibacterial chemicals and IgA. The efficiency of colonisation essentially depends on adherence, or adhesion (both words being synonyms), to the epithelial cells and/or to the extracellular matrix. Thanks to colonisation of the epithelia the bacteria can indeed « 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, [and] establish them selves within a host. »

Definition of bacterial adhesion

The adhesion of bacteria is mediated by surface structures, named adhesins, which interact with components of the eukaryotic cell surface or of the extracellular matrix, named receptors. Today dozens of bacterial adhesins, different receptors and three main types of adhesin-receptor interactions are described : firstly, protein adhesin structure, called lectin and carbohydrate receptor, secondly, protein adhesin and protein receptor and thirdly, hydrophobin adhesin and hydrophobin receptor.

Regarding the first type of interaction, lectins are structurally defined adhesins of Gram negative and Gram positive bacteria and are classified in several groups by their haemagglutination properties, ultrastructure and receptor specificity. Their receptors are of two types : firstly, the carbohydrate moieties of glycoproteins or glycolipids of the cell membrane, which mediate cell to cell contact or serve as part of a host signal transduction mechanism, and secondly, the carbohydrate moieties of components of the extracellular matrix, present either in either soluble, immobilised or tissue-bound form.

As for the second type, bacterial adhesion can also be mediated by proteins that either are component of the outer membrane of Gram negative bacteria or of the cytoplasmic membrane of wall-free bacteria, or are associated to the peptidoglycan of Gram positive bacteria. Their receptors are amino acid sequences of proteins that are also components of the eukaryotic cell cytoplasmic membrane or of the extracellular matrix and play the same roles as previously described.

The third type of bacterial adhesion is mediated by components named hydrophobins that promote cell surface hydrophobicity, frequently bacterial lipids and eukaryotic glycolipids or hydrophobic moieties of proteins. Their mechanism of action would be to overcome the repulsive forces that separate negatively charged particles and to mediate weak reversible adhesion. « Real » adhesion would then follow.

This second lecture is based upon the description of the following three types of bacterial adhesins : (i) fimbrial and afimbrial adhesins of Gram negative bacteria whose carbohydrate or protein receptors are associated with the eukaryotic cell cytoplasmic membrane ; (ii) adhesins of Gram positive bacteria whose protein receptors are components of the extracellular matrix ; and (iii) adhesins of Gram positive and Gram negative bacteria that bind to protein receptors present in the cytoplasmic membrane of the eukaryotic cell and can promote subsequent invasion of these eukaryotic cells.

FIMBRIAL AND AFIMBRIAL ADHESINS OF GRAM NEGATIVE BACTERIA

Early studies and classifications

Though Gram negative bacteria adhesion was actually discovered at the beginning of the 20th century when G. Guyot observed that some *Escherichia coli* strains cause haemagglutination, the phenomenon was extensively studied only from the 1950s onwards by, among others, J.-P. Duguid and his collaborators in a series of works published between 1955 and 1979. Duguid and his collaborators described different electron microscope visible appendices responsible for the haemagglutination. They named these *fimbriae* (thread, fibre or fringe in Latin). They also proposed the first real classification of bacterial adhesins based on their haemaglutinating properties and on their ultrastructure under electron microscopy : the haemagglutinins of the first group cause a D-mannose-sensitive haemagglutination (0.5% w/v) and have a fimbrial structure ; the haemagglutinins of the second group cause mannose-resistant haemagglutination and are also fimbriae ; the haemagglutinins of the third group also cause mannose-resistant haemagglutination, but are not fimbriae and form no visible structures under electron microscope. The mannose-sensitive haemagglutinins were named type 1 fimbriae while the numerous fimbrial and afimbrial mannose-resistant haemagglutinins receive different specific names during the following years, according to many different criteria. This classification scheme does not, however, include adhesins with no haemagglutinating properties.

In parallel with the work of Duguid and his collaborators, and, later on, other classification systems were independently proposed. The classification, proposed in 1990 by F. and I. Orskov, for the appendices of Gram negative bacteria involved in adhesion is based upon their detailed ultrastructure and molecular composition, irrespective of their haemagglutinating activity. Four categories were proposed by the Orskovs. The first category comprises rigid, thick and peritrichous fimbriae (5-7 nm) with an axial hole and the second category is composed of flexible, thin and peritrichous fimbriae (2-3 nm) with no axial hole. The former fimbriae include the mannose-sensitive, or type 1, haemagglutinins of Duguid and collaborators and several mannose-resistant haemagglutins. The latter fimbriae are also named *fibrillae* (thin, small fibres in Latin). The third category includes even more flexible and thinner, highly aggregated fimbriae, named *curli* (curve, spiral, loop, especially for hair, in Latin). The fourth category comprises flexible, moderately thick (4-6 nm), bundle- or rope-forming and polarly distributed fimbriae corresponding to the so-called type 4 fimbriae of previous classifications. This classification does not, however, include any adhesin not forming appendices at the bacterial cell surface, like the afimbrial haemagglutinins of Duguid.

The three following categories of Gram negative bacterial adhesins will be described : categories 1 and 2 of classical fimbriae and of the Orskovs' fibrillae, with emphasis on the P fimbriae of *Escherichia coli* and one group of afimbrial adhesins with mannose-resistant haemagglutinating properties, the Afa family. However, the curli and the type 4 fimbriae will not be presented, because their role in the pathogenesis of bacterial infections is not well understood and not enough molecular information is available.

Structure-function relationship

i) P fimbriae

One of the most studied groups of fimbriae are the *E. coli* P fimbriae, so called because they cause mannose-resistant haemagglutination of human erythrocytes belonging to the P blood group. The role of P fimbriae in the virulence of human uropathogenic *Escherichia coli* is considered important and specific to the development of urinary tract infections, in particular pyelonephritis. Their second name is therefore Pap fimbriae after « Pyelonephritis-associated Pili ». P fimbriae may also play a role in several extra-intestinal infections in animals, such as urinary tract infections, especially in dogs and cats, or septicaemia, especially in calves, piglets and poultry. The basis of the shaft of all fimbriae and fibrillae is a protein called the major subunit or pilin protein, with a molecular weight of between 15 and 20 kDa, present in several hundred or thousand copies. Several other protein components, named minor subunits, are present at the top or at the base of the structure in only a few copies, sometimes only one copy.

The one thousand copies of the major subunit of the P fimbriae, a 19.5 kDa protein named PapA, are arranged in a right-handed helix containing 3.3 subunits per turn, starting from the outer membrane and elongating toward the external world with a total length of 1 μm. This 7 nm wide cylinder is hollow with an axial hole of 1.5 to 2 nm.

One important minor subunit of the fimbrial structure is the so-called « anchor protein », or PapC subunit, which is embedded inside the external layer of the outer membrane and represents the real base to which the fimbriae are attached. To ensure a firm and irreversible anchorage, other minor subunits, such as PapH, play a role in consolidation. The anchor protein also plays an important role in the biogenesis of the fimbriae allowing passage of the subunits in the right order, so that they are placed in correct position within the fimbriae. For this reason, the anchor protein is also often named the « usher protein ». The P fimbriae are topped by a 40-80 nm long, 2 nm wide distinct structure called the « tip fibrillum », composed of a few other minor subunits. The most important one, PapE, is present in several copies forming a flexible helix without any axial hole. At the very tip of this fibrillum is present a specific minor subunit, the PapG subunit, which is the real adhesin i.e. the protein responsible for interaction with the carbohydrate receptor. The adhesin PapG subunit is a two-domain protein, with the NH2 terminus participating in receptor recognition and with the COOH terminus being required for the incorporation, in the right place and under the right conformation, of the adhesin subunit on the fimbrial structure. Mutants defective only in the production of the adhesin minor subunit can not adhere to the host cells and can not colonise the host tissues, though they do produce otherwise intact fimbriae.

ii) Other « classical » fimbriae and fibrillae

Other Gram negative bacterial fimbriae and fibrillae differ in their overall structures, notably in the number

Table 1. Examples of fimbrial and fibrillal adhesins of *Escherichia coli*

Name	Type	Pathogenic *E. coli*	Adhesin	Major receptor moiety	Molecule
F1	*fimbriae*	all	FimH	mannose, methyl-α-D-mannose	
F2	*fimbriae*	enterotoxigenic (human)	CfaB (pilin)	NeuAc	Glycoprotein
F4	*fibrillae*	enterotoxigenic (porcine)	FaeG (pilin)	NeuGc-(α2-3)-Gal- (β1-4)-Glc, Fuc-(α1-2)-Gal-(β1-3/4)-GlcNAc	
F5	*fibrillae*	enterotoxigenic (bovine, porcine)	FanC (pilin)	N-glycolyl neuraminic acid	Glycolipid
F17	*fibrillae*	various (bovine + avian, human, porcine)	F17G	GlcNAc	
P (Pap,Prs)	*fimbriae*	uropathogenic (human, canine, feline)	PapG, PrsG	Gal-(α1-4)-Gal	Glycolipid
S (Sfa)	*fimbriae*	Uropathogenic + invasive (human, animal)	SfaS	NeuAc-(α2-3)-Gal- (β1-3)-GalNAc	Glycosphingolipid
Afa (Afa, Nfa, Dr, F1845, M)	afimbrial	Uropathogenic + invasive (human)	AfaE, NfaA, DraA, DaaE, BmaE	SCR3 domain (different peptide sequences)	DAF (Decay Accelerating Factor) or CD55

of minor subunits, in the structure or absence of a tip fibrillum, in the adhesin subunit, and in the length of the fimbriae. The fibrillae differ from fimbriae by being thinner (only 2-3 nm) and having no axial hole. Let us briefly introduce some of the fimbriae and fibrillae of different pathogenic strains of *Escherichia coli* (table 1).

The S fimbriae produced by uropathogenic and invasive *E. coli* in humans and in different animal species are very similar to the P fimbriae, with fewer subunits. They carry a tip fibrillum very similar to that of the P fimbriae and this is topped with one adhesin subunit.

The structure of the type 1 or F1 fimbriae is also very similar to the structure of the P and S fimbriae, but the tip fibrillum is shorter, extending only 16 nm. Type 1 fimbriae are produced by virtually all strains of *E. coli* and by many other enterobacteriaceae, and their role in virulence is highly questionable.

The F17 fibrillae have been associated with diarrhoeic and invasive *E. coli*, mainly in cattle and sheep, and with human uropathogenic *E. coli*, but are also produced by several non-pathogenic human and animal *E. coli* strains. F17 fibrillae are much simpler than P, S and type 1 fimbriae since they are composed of only two subunits : the structural major subunit, F17A, with intercalated functional copies of the adhesin minor subunit, F17G.

In other fimbriae and fibrillae, there exists no distinct adhesin minor subunit and the adhesion function is present in a domain of the major subunit, with either only the fimbrial top major subunit exposing the adhesive domain (as on F2 fimbriae of human enterotoxigenic *E. coli*), or several structural major subunits (as on F4 or K88 fibrillae of porcine enterotoxigenic *E. coli*), or all structural major subunits (as on F5 or K99 fibrillae of bovine and porcine enterotoxigenic *E. coli*). These composite major subunit-adhesin proteins structurally resemble the classical two domain adhesin subunits of other fimbriae and fibrillae. The F5 fibrillae are also peculiar in presenting different minor subunits intercalated between stretches of major subunits.

iii) Afimbrial adhesins

The Afa family of *E. coli* adhesins (table 1) includes afimbrial structures, such as the Afa adhesins themselves and the M haemagglutinin, or small fibrillal structures, such as the Dr haemagglutinin and the F1845 fibrillae, or capsule-like structures surrounding the bacterial cell, such as the Nfa adhesins. The members of the Afa family are expressed by human uropathogenic and diarrhoea-associated *E. coli*.

All afimbrial and fibrillal structures of the family are attached to an anchor-usher protein (AfaC for instance). The actual afimbrial adhesins of the family comprise two additional proteins : a subunit with the function of an adhesin (AfaE) and a second subunit with the function of an adhesin/invasin (AfaD). In the members of the family with a fibrillal structure all copies of the major subunit also possess a function of adhesion. Their actual structure is dictated by the gene coding for the adhesin subunit or for the major subunit-adhesin of the fibrillae, as shown in complementation and chimaeric construction experiments.

The Afa and M afimbrial adhesins actually resemble truncated fimbriae or fibrillae after loss of the major subunit, with an anchor protein and one or two other subunits. But instead of being a regression of an elaborated structure they might be ancestors of fimbriae and fibrillae. One can imagine an evolution from afimbrial adhesins, toward fibrillal adhesins in

which the major subunits also function as adhesin, then toward structures with only the top major subunit functioning as an adhesin, followed by the specialisation of this protein as a specific adhesin subunit. Meanwhile fibrillae evolve as fimbriae although some keep a more or less developed tip fibrillum.

But how does a bacterium build up such elaborated structures ?

Biogenesis

Of the four distinct mechanisms of biogenesis of « classical » fimbriae and fibrillae, the « chaperone-usher pathway » is the most studied and the best understood and will be described with the P fimbriae as the leading example. The biogenesis of afimbrial adhesins also follows the « chaperone-usher pathway ».

The different major and minor subunits reach the cytoplasmic membrane after translation of messenger RNA. They cross the cytoplasmic membrane utilising the general secretion (Sec) system, thanks to their amino-terminal signal peptide that is cleaved after completion of the crossing. Though the subunits are now in the periplasm, they remain attached to the cytoplasmic membrane by their hydrophobic carboxy-terminal sequence, and are fully liberated only during interaction with the so-called chaperone proteins, such as PapD, which have an immunoglobulin-like folding. After release from the cytoplasmic membrane each subunit undergoes an initial folding into an assembly competent conformation by exposing an interactive site which is responsible for their polymerisation and assembly. Only binding with the chaperone prevents the immediate polymerisation in the periplasm by masking this interactive site. If no interaction with the chaperone occurs, the subunits polymerise inside the periplasm and are degraded. The stable chaperone-subunit complex moves through the periplasm toward the outer membrane and reaches the site where the anchor protein subunits, such as PapC, form an oligomeric complex : the « outer membrane usher ». After the binding of the complex chaperone-subunit, the outer membrane usher structure opens a 2 to 3 nm wide channel through which the subunits are translocated. The subunits polymerise during the translocation process at the surface of the outer membrane of the bacteria. The energy necessary for the final assembly of the different subunits is derived from the favourable entropy generated by the interactions between these different subunits in the mature fimbriae. There is no need for any enzymatic activity or substrate degradation to produce energy to build up the structure.

In the biogenesis of the P fimbriae the translocation of the different subunits is highly ordered, with translocation of the tip adhesin, PapG, being followed by that of the other tip fibrillum subunits, such as PapF, PapE and PapK, that of the major subunit PapA, and that of the minor subunits, such as PapH, at the base of the fimbriae. The order appears to be the consequence of both the relative concentrations of the different subunits in the periplasm at the time and the relative binding affinity of each chaperone-subunit complex to the outer membrane usher.

Interaction with the host receptors

The receptors of fimbriae and fibrillae are the carbohydrate moiety of glycoproteins or glycolipids, while the receptors of the afimbrial adhesins are peptide sequences.

i) Fimbriae and fibrillae

The first basis of carbohydrate receptor identity or primary sugar specificity, is identified to the simplest carbohydrate structure that best inhibits bacterial adhesion: hexoses (such as D-mannose for type 1 fimbriae), methylpentoses, acetylhexosamines, sialic acid, etc. This primary sugar specificity is the result of a macroevolution occurring over a millennial time frame, probably since the first colonisation of a host by a bacterium, leading to gross host and tissue tropisms. But the actual *in vivo* situation is a little more complex, since within lectin-adhesins belonging to the same family and possessing the same primary sugar specificity, subtle differences in the binding of different oligosaccharides are often observed leading to fine sugar specificity. This microevolution of receptor specificity has occurred within a shorter time frame and simultaneously to an intra-bacterial species evolution and diversification of adhesins leading to fine host and tissue tropisms. The macro- and microevolutions are consequences of phenotypic and genetic modifications of the adhesion molecules. Phenotypic variation is due to conformational changes of the adhesin itself under the influence of other mutating proteins involved in its presentation on the bacterial surface. Genetic variation is more complex and can be achieved by horizontal transfer of foreign genes, allelic variation and/or gene rearrangement.

Table 1 summarises the macroevolution of receptor specificity of the fimbriae and fibrillae of *E. coli* described earlier : F1, F2, F4, F5 and the F17, P and S families. Already this macrospecificity can tell us a lot about the host specificity of these pathogen *E. coli*. For example, the F5-producing enterotoxigenic *E. coli* are specific to newborn piglets and calves, because the receptor, the N-glycolylneuraminic acid, of F5 fibrillae is found on intestinal cells of newborn piglets and calves, but disappears as the animal ages. So the receptivity is restricted to the newborn period. Moreover the glycolipid receptor is not present on the human intestinal epithelial cells, and F5-producing *E. coli* are never isolated from diarrheic humans for this simple reason. Let us take P fimbriae, once more, as a second example.

The primary sugar specificity of the PapG adhesins is the moiety α-D-galactopyranosyl-(1-4)-β-D-galactopyranoside, or Gal (α1-4) Gal, present in a globoseries of glycoplipids, which correspond to the P blood group.

At least three variants of the PapG adhesin protein have been described to date : PapG1, PapG2 and PapG3. The fine sugar specificities of these three adhesins can be summarised as follows : Gal (α1-4) Gal (β1-4) Glucose, or globotriaose, or GbO3 for the PapG1 adhesin ; GalNAc (β1-3) Gal (α1-4) Gal (β1-4) Glc, or globotetraose, or GbO4 for the PapG2 adhesin ; GalNAc (α1-3) GalNAc (β1-3) Gal (α1-4) Gal (β1-4) Glc, or globopentaose, or GbO5 for the PapG3 adhesin.

Their fine receptor specificity is reflected in the *in vivo* situation to some extent. The PapG1 and PapG2 receptors are present on the epithelial cells of the urinary tract of humans (GbO3 in the bladder and GbO4 in the kidney) while the PapG3 receptor is primarily present in the urinary tract of animals, especially dogs and cats. If we forget the PapG1 adhesin which is very rare, the PapG2 adhesin

is produced at first by human uropathogenic *E. coli* and the PapG3 by animal uropathogenic *E. coli*. Even finer variations exist since the PapG3 adhesin of the P fimbriae named F165-1 recognises a slightly different receptor than the original PapG3 adhesin and agglutinates porcine and bovine erythrocytes that the original PapG3 adhesin does not. This PapG3 adhesin variant is produced by many pathogenic *E. coli* in bovines and pigs. This example of the microevolution of the PapG adhesins probably also illustrates a host-pathogen co-evolution.

ii) Afimbrial adhesins

Another example of variation in receptor specificity is the family Afa of afimbrial adhesins (table 1). Members of the Afa family recognise the Decay-Accelerating Factor (DAF or CD55), as a receptor. DAF is a 70 kDa glycoprotein widely distributed on human haemopoietic, endothelial, intestinal and urinary cells. The urinary and intestinal localisations correspond to the tissue tropisms of *E. coli* strains, producing one or other of these adhesins.

The protein moiety of DAF, or the Dr blood group antigen, is exposed at the cell surface by its NH2 terminus, consisting of four short consensus repeat (SCR) domains. The COOH terminus is the site of attachment of a glycosylphosphatidylinositol anchor which is attached to the eukaryotic cell membrane. The Afa-I, Afa-III, Dr and F1845 adhesins bind to the SCR3 domain. Other adhesins of the family may recognise the SCR4 domain instead. The physiological role of DAF and, more particularly, of the SCR3 domain is to bind the C3b or C4b components of the complement cascade, therefore preventing the assembly of C3 convertase and erythrocyte lysis by complement. It can, therefore, be suggested that the binding of any Afa adhesin to the SCR3 domain of DAF prevents the fixation of C3b or C4b and consequently allows the pursuit of the cascade, leading to complement-mediated tissue damages. The *E. coli* could then bind more easily to the newly exposed extracellular matrix via other adhesins produced simultaneously or in sequence. DAF-like molecules of the tissues of animal species show biochemical differences, which explains that *E. coli* producing adhesins of the Afa family have been isolated from humans only, until recently.

iii) The story of the afa-8 variant

An investigation was undertaken by DNA colony hybridisation using radioactively labelled gene probes derived by PCR from the conserved family genes coding for the usher and for the chaperone proteins of the Afa-III variant (Mainil *et al.*, 1997). During this investigation, positive signals were obtained for the first time amongst a collection of necrotoxigenic *E. coli*, first from cattle and then from pigs and humans. If the signal was less intense than for the positive control, the hybridisation study was repeated several times with the same results. On the other hand a family PCR assay targeting the same genes gave only negative results, leading to the conclusion that new variants of the Afa family were present in those necrotoxigenic strains.

A new Afa variant was indeed identified by Dr Chantal Le Bouguénec from the Pasteur Institute in Paris, in one of the bovine necrotoxigenic *E. coli* and named Afa-VIII (Lalioui *et al.*, 1999). Later, three PhD students in my laboratory, Joël Gérardin, Sigrid Van Bost and Philippe Stordeur showed that Afa-VIII is so far the only member of the family present amongst animal *E. coli*. Afa-VIII is produced by *E. coli* strains associated with intestinal and invasive clinical conditions in different mammalian species (particularly bovines), and in poultry. It is also produced by some human *E. coli* strains associated with urinary tract infections, under the name of M haemagglutinin. In contrast with the « human » members of the family, the newly described animal AfaE-VIII adhesin does not recognise the DAF molecule as a receptor. Its receptor might be related to the glycophorin A^M that is present on the human red blood cells belonging to the M group and that is recognised by the M haemagglutinin, the only member of the Afa family to which the Afa-VIII adhesin is closely related. This difference in receptor specificity is also reflected by the absence of binding of the Afa-$VIII^+$ strains to Hep-II and HeLa cells to which the strains carrying other variants of the family adhere in a diffuse pattern. The Afa-$VIII^+$ strains adhere only to the MDCK I cell line, but not even to the MDCK II cell line.

ADHESINS OF GRAM POSITIVE BACTERIA

Classification

In contrast with the adhesins of Gram negative bacteria, the classification of the adhesins of Gram positive bacteria is based upon their association to the bacterial wall and not upon their morphology. Four classes are therefore described : covalent linkage to the peptidoglycan by a transpeptidation reaction (also named cell wall-anchoring mechanism) ; cytoplasmic membrane anchorage of a hydrophobic membrane-spanning domain (or transmembrane mechanism) ; association with surface proteins ; and association with surface glycolipids. Many adhesins of Gram positive bacteria are amorphous, but some form distinct structures protruding from the bacterial surface, whose morphology varies from short filaments, called « fuzz », to long filaments, like fimbriae. Some adhesins are sparsely distributed on the cell surface, while others are more densely distributed. Amongst the Gram positive bacteria the adhesins covalently linked to the peptidoglycan are the best known. Examples of such adhesins are the internalins of *Listeria monocytogenes*, which will be described in the next chapter, and the fibronectin-binding family, which will be described in the following section.

Gram positive bacteria seem, more so than Gram negative bacteria, to carry « *Microbial Surface Components Recognising Adhesive Matrix Molecules* » or MSCRAMMs, which mediate adhesion to components of the extracellular matrix: fibronectin, collagen, fibrinogen, vitronectin, laminin, elastin… Some of these MSCRAMMs bind indifferently to the soluble, the immobilised or the tissue-bound form of the matrix component, while others exclusively bind to only one form leading, to specificity in their pathogenicity. The principal function of tissue-bound extra-cellular matrix components, for instance, is to serve as adherence substrate for the host cells. The extracellular matrix-binding bacteria have exploited this physiological function to come into very close contact with the host cells so that they, in turn, can colonise and invade tissues, using other adhesins. The tissue-bound form of extra-cellular matrix components thus serves as a bridge between the bacteria and

the physiological host cell receptor for that component, in comparison with the direct attachment of Gram negative bacteria to the host cells.

The fibronectin-binding family

The large family of fibronectin-binding proteins of Gram positive bacteria comprises several wall-anchored adhesins, which interact with one amino-acid moiety of fibronectin and not with its carbohydrate moiety. Fibronectin is bound to its cell receptor, named integrin, and therefore forms a bridge between the bacterium and the host cell. This family includes, among others, the F1 and F2 proteins of Lancefield group A streptococci, the FnB-related proteins of Lancefield group C streptococci and of staphylococci and the GfbA protein of Lancefield group G streptococci. These different proteins bind with very high specificity and affinity to either the soluble or the immobilised fibronectins.

The fibronectin-binding adhesins are encoded by a single structural gene but show a very much elaborated structure-function relationship. The description of the F1 protein of *Streptococcus pyogenes* will serve as a general example. The amino-terminus of F1 begins with a signal sequence typical of exported Gram positive proteins. Then follows a long domain (U) containing unique sequences that are divergent between the members of the family, but whose function is still unknown. The function of the following domain (RD1), which consists of several tandem repeats within an A-B sequence motif, also remains a mystery. The structure of the carboxy terminus consists of a short, positively charged, sequence (C) that remains inside the cytoplasm, a hydrophobic domain (M), possibly involved in the interaction with the bacterial cytoplasmic membrane and a proline and lysine rich domain (W), which plays an important role in the interaction with and linkage to the bacteria peptidoglycan. A consensus sequence, the LPXTG sequence, for Leu-Pro-X-Thr-Gly, located between the W and M domains, also plays a crucial role in the linkage to the peptidoglycan.

The signature feature of the family, the RD2 domain, consists of an amino acid motif that can be repeated up to 6 times in tandem. These repeats vary from a few to several hundred amino acids, with variations not only between bacterial species, but also between strains within the same bacterial species. The signature domain of F1 consists of a 32 to 44 amino acid motif. There exists strong evidence that this signature domain is primarily implicated in the binding to fibronectin. In addition, a short amino acid sequence just upstream of the RD2 domain of F1 (UR) also participates in the binding of this adhesin to fibronectin. The UR domain has been found in other adhesins of the family but can be more distantly located from the RD2 domain. The RD2 and UR domains bind to different peptide sequences of fibronectin.

After the translation of the messenger RNA, the biogenesis of wall-anchored adhesins begins with their secretion through the cytoplasmic membrane via the general secretion Sec system, thanks to their $-NH_2$ terminal signal peptide, or S domain. But they are temporarily retained in the cytoplasmic membrane by their –COOH hydrophobic transmembrane M domain and the short charged cytoplasmic tail or C domain. The next step is the cleavage of the membrane-bound protein at the height of the consensus sequence LPXTG. The cleavage occurs between the Thr and the Gly, and the enzyme responsible is named « sortase ». The new -COOH terminal Thr amino acid at the end of the freed W domain is subsequently linked to a nascent muramyl-peptide, a constituent of the peptidoglycan polymer, by a transpeptidase reaction. These adhesins are therefore covalently linked to the peptidoglycan and can be released only after enzymatic cleavage. Meanwhile, the free NH_2 terminus of the protein after cleavage of the signal sequence has progressively expanded through the network formed by the peptidoglycan, a journey facilitated by the formation of random coils by the proline and glycine-rich stretch of the W domain.

INTERNALINS, INVASINS AND INTIMIN

Post-adhesion events

Years ago, bacterial adhesion was considered as merely a static process involving the binding of preformed molecules on the surface of the bacteria and of the eukaryotic cells, not requiring any of the partners to be viable. Results of *in vitro* experiments with bacteria or semi-purified fimbriae, and with formalised eukaryotic tissue fragments, supported this view. Bacterial adhesion was considered as only a prerequisite for the release of toxins close to the host target cells. Today, bacterial adhesion is seen as a dynamic process that induces post-adhesion events in and cross-talks between both partners. The results of these, in addition to the production of toxins causing membrane and metabolism perturbations, can be modifications of the cytoskeleton leading to invasion of the host cell by the adherent bacteria and/or regulation of the production of metabolites, including cytokines, by the host cells. The action of bacterial toxins on the host cells is the subject of the next lecture. The following sections will describe the invasion of non-phagocytic host cells as a post-adhesion event. However, the regulation of the production of cytokines as a post-adhesion consequence by activation or repression of their encoding genes following different cascades will not be discussed.

Non-phagocytic cell invasion

Since non-professional phagocytes do not usually take up large particles, bacteria, or other pathogens, they must promote their own entry to gain access to the cytoplasm of these cells. Three actin-dependent mechanisms of invasion of non-phagocytic eukaryotic cells by bacteria have been described so far : (i) the zipper mechanism by *Listeria* and *Yersinia* ; (ii) the trigger mechanism by *Shigella* and *Salmonella* ; (iii) the invasome mechanism by *Bartonella*. The zipper mechanism represents the best example of an invasion process depending solely on a cascade of events in the eukaryotic cell initiated by the adhesion of bacteria. The trigger mechanism represents a more elaborate cross-talk between bacteria and eukaryotic cells. This chapter will end with the description of the intimin binding of some pathogenic strains of *Escherichia coli* to the enterocyte cytoplasmic membrane involving similar cross-talks between bacteria and host cells.

The zipper mechanism

The zipper mechanism is very similar to the receptor-mediated uptake

of particles by phagocytic cells. The first step is a specific high-affinity tight binding of bacterial cell surface components to their receptors on the eukaryotic cells. The interaction between the bacterial adhesin and the cellular receptor directly induces modest and local polymerisation and reorganisation of actin filaments at the cytoplasmic membrane, but only in the immediate neighbourhood of the bacterial attachment zone. This allows the bacteria to be taken up in a tight phagosome. This internalisation can be inhibited by microfilament-disrupting drugs independently of the adhesion.

Different bacterial wall-anchored adhesins, called internalins, have been identified that allow the Gram positive bacterium, *Listeria monocytogenes,* to zipper into eukaryotic cells. Internalin A (InlA) is an 88kDa surface protein belonging to a family characterised by the presence of Leu-rich repeats. Mutagenesis experiments have demonstrated that InlA is necessary for invasion of intestinal epithelial cells in culture through its interaction with the host receptor, the E-cadherins. The classical cadherins consist of transmembrane homodimeric glycoproteins, which are involved in cell to cell interactions at the height of the adherens junctions of polarised cells, such as enterocytes, where they establish associations with the cadherins of the adjacent cell. The external domain of E-cadherin is necessary for the binding of InlA, while the intracytoplasmic domain is necessary for internalisation of *Listeria*. The interaction between InlA and E-cadherin is species-specific and involves recognition of a proline residue of the extracellular domain of E-cadherin. But other adhesins may take up the role if InlA is mutated. Internalin B (InlB), for instance, is a 65 kDa protein that is encoded by a gene located in the same operon as the InlA-encoding gene and is responsible for the invasion of cell types other than enterocytes. The host cell receptor is the same molecule that serves as the receptor for the C1q complement component (gC1q-R). In addition, *Listeria* harbours at least five other *inl*-like genes coding for proteins containing Leu-rich repeats that may also function as internalins. This diversity in adhesins perhaps reflects the wide potential of cellular targets of *Listeria*.

The adhesins of the Gram negative bacteria, *Yersinia* sp., are outer membrane proteins, named invasins. *Yersinia* invasins bind tightly to the β1 integrins that mediate adhesion of eukaryotic cells to each other and to the extracellular matrix. The integrins are heterodimeric proteins composed of two non-covalently associated subunits, designated α and β. Sixteen different α variants and eight different β variants have been identified in mammals to date. The classification of integrins is based upon the identity of the β subunit and β1 integrins are present on epithelial and mesenchymal cells. Both subunits of the integrins are attached to the cytoplasmic membrane by a transmembrane hydrophobic domain located near the NH2 termini. The two extended heads of the β integrin subunits contain the Arg-Gly-Asp (RDG) motif serving as a ligand-binding or adhesin-binding domain.

Both E-cadherins and β1 integrins interact with the actin cytoskeleton via the binding of their cytoplasmic NH2 terminus to adhesion plaque proteins (α-actinin, vinculin, talin, catenin, and others), which themselves are linked to the actin network of the cytoskeleton. These interactions could represent the link between the internalins and invasins and the cytoskeletal changes that follow *Listeria* or *Yersinia* binding and result in their internalisation. Both these examples actually illustrate bacterial exploitation of mechanisms normally involved in host cell adhesion. Engagement of these receptors by their physiological ligands induces signalling cascades that result in the strengthening of the cell to cell and cell to extra-cellular matrix contacts and in cellular differentiation. So, when bacterial surface protein adhesins, such as internalins and invasins, engage their receptors, the host cells respond as they would physiologically : recruiting cytoskeletal elements to the site of adhesion of the bacteria and attempting to strengthen the attachment to other cells. However, since the bacterium is much smaller compared to the responding cell, the attempt by the cell to spread against the bacterial surface quickly results in engulfment of the bacterial cell. The zipper mechanism is thus an easy non-dramatic hardly noticeable small scale mechanism of entry by a bacterium into a eukaryotic host cell.

The trigger mechanism

In the trigger mechanism, the interaction between the bacterial adhesin and the cellular receptor induces the translocation of bacterial effectors into the eukaryotic cell through a type III secretion system. This triggers a cascade of reactions, including activation of small GTPase proteins, which regulate the actin cytoskeleton. Like the zipper mechanism, the trigger mechanism is thus actin-dependent, but requires a much more complicated type of bacterial machinery to induce rapid intracellular polymerisation and reorganisation of actin filaments on a much larger scale. Explosive actin filament polymerisation under the cytoplasmic membrane throws up large membrane extensions, or membrane ruffles that fold over and trap the bacteria and quite a large area of extra cellular environment in an intracellular membrane-bound pocket. This process is called macropinocytosis. Although sharing common traits with the zipper mechanism, the trigger mechanism is thus a much more dramatic, sophisticated and persuasive mechanism of entry of a bacterium into a eukaryotic host cell.

Type III secretion systems represent a mechanism of direct injection, or translocation, of bacterial effectors, usually proteins, into the cytoplasm of the eukaryotic target cells. In the case of the trigger mechanism of cell invasion, as well as with the intimate binding of *E. coli* to target cells, which will be described in the next section, the bacterial effectors interact with the regulation cascades of cell cytoskeleton integrity causing wide-scale rearrangements. Moreover, each bacterial species produces its own specific type III secretion machinery, although some of them clearly show some degree of structural and/or functional homology. The genes coding for the type III secretion system and for the bacterial effectors can be grouped together on a pathogenicity island.

The trigger mechanisms of the *Shigella* species invasion of intestinal epithelial and M cells involves proteins whose encoding genes are carried on a 220 kb virulence plasmid. This virulence plasmid, carrying genes coding for the type III secretion system becomes active when the bacteria come into contact with the host cells by mobilisation of proteins already existing in the cytoplasm of the bacterial cells : the Ipa, after « Invasion plasmid antigens » ; or within the bacterial membranes : the Mxi/Spa, after « Membrane expression of Ipa » and « Surface presentation of antigens ». Ipa are the translo-

cated bacterial effectors, whereas the MXi/Spa form the type III secretory apparatus. IpaB and IpaC proteins also appear to be responsible for the initial attachment of the bacteria to the eukaryotic cell membrane and α5β1 integrin might be the receptor of the complex. The Ipa complex interacts on the outside of the eukaryotic cell and activates signal transduction pathways that are responsible for the cytoskeletal rearrangements and membrane ruffling. The ruffles extend actin-rich membrane projections near the site of attachment of the bacterium, which contain actin-bundling protein fimbrin (plastin). The ruffles also contain, of course, several adhesion plaque proteins, such as talin, α-actinin, paxillin, vinculin and the focal adhesion kinase (FAK), with which the β subunits of the integrin receptors are linked by their cytoplasmic domain. The regulatory signals responsible for the actin rearrangements include activation of the low molecular weight GTPase Rho, a central regulator of the eukaryotic cell metabolism including the cytoskeleton physiology. Inhibitors of Rho block cell invasion by *Shigella*.

The *Salmonella* species are also facultative intracellular bacteria that can enter several cell types by the trigger mechanism. The target cells are enterocytes and macrophages, into which *Salmonella* prefer to enter by forced rather than by classical phagocytosis. *In vitro* studies have identified several host signalling pathways that may be responsible for the cytoskeletal rearrangements upon interaction of *Salmonella* with the host cell cytoplasmic membrane. The importance of each pathway varies according to cell type. Unfortunately the bacterial proteins that are responsible for the activation of the host cell pathways have not yet been formally identified, nor has any cellular receptor, although several indispensable proteins and genes and proteins have been identified following mutagenesis experiments.

The intimate binding

Intimin is a 94 kDa outer membrane protein of *Escherichia coli* that has some genetic and functional homology to the invasin of *Yersinia*. The main difference between *E. coli* and *Yersinia* is that the former does not typically invade the eukaryotic cell after adhering to its surface. Intimin is present on enteropathogenic and enterohaemorrhagic *E. coli* strains. These produce the so-called attaching and effacing lesions with effacement of the enterocyte microvilli, compared with the intact epithelium, and intimate attachment to the cytoplasmic membrane of the enterocyte.

The receptor of intimin represents the only known example of bacteria translocating its own receptor into the eukaryotic host cell membrane using a type III-secretion system. The intimin receptor has been named Tir protein, for Translocated intimin receptor. In the eukaryotic cell, Tir becomes phosphorylated on tyrosin residues and integrated into the host cell cytoplasmic membrane. Tir exhibits a hairpin conformation with the extracellular loop binding to intimin and the NH2- and COOH-terminal domains projecting into the host cell cytoplasm and interacting with adhesion plaque proteins, particularly α actinin and talin. It therefore indirectly interacts with the actin network of the cytoskeleton, just as the internalins and invasins of *Listeria* and *Yersinia* do. A second putative intimin receptor is β1 integrin, but this idea is still very speculative, though nevertheless a reminder of the homology to *Yersinia* invasins.

In contrast with *Yersinia*, enteropathogenic and enterohaemorrhagic *E. coli* cause quite dramatic modifications of the cell skeleton with retraction and effacement of the microvilli of the enterocytes and production of a pedestal of actin filaments under the zone of bacterial attachment by three different mechanisms : intimin-Tir binding, intimin-β1 integrin binding and type III-secreted effectors acting directly and more extensively on the cytoskeleton. The classical final image is an intimately adhering bacterium resting on the pedestal, but the scale of the changes induced can vary from strain to strain. And although uncommon, images of internalised bacteria can be observed both *in vitro* and *in vivo* with some strains.

OF ADHESINS AND MEN

Since adhesion of bacteria to host cells and tissues was recognised as one of the important stages in the development of bacterial infectious diseases, man has tried to use that property in order to fight pathogen bacteria. Over the course of time, different strategies have been developed and used as prophylactic or therapeutic measures, with variable success. The most interesting ones are the adhesin-based vaccines, the saturation of the binding sites of the bacterial adhesins by analogs of the receptors and the saturation of the receptors of the target cells and tissues by analogs of adhesins. Major drawbacks of any anti-adhesion strategy are as follows : the production of multiple adhesins by bacterial clones, the fact that all adhesins are not necessarily produced at one time at one site and the existence of antigenic and receptor variants of the parental adhesin in different bacterial clones. In each case, a small number of bacteria carrying a minor adhesin may survive the treatment directed against the predominant variant. There is only a need for the presence of receptors, even in small numbers, of this minor adhesin, to avoid elimination of all the bacteria by mechanical defences.

In the beginning, most efforts in preventing the development of diseases were directed towards the production of anti-adhesin antibodies, with major success especially against enteric colibacillosis in neonates, calves and piglets. But this situation was peculiar in various ways : the typical virulence properties of enterotoxigenic *E. coli* are very simple, with the production of only a few fimbrial adhesins and toxins ; the fimbrial adhesins are involved in the very early stages of the disease ; the number of different adhesins remains amazingly low with very little antigenic variation, if any ; all fimbrial adhesins are very good immunogens ; finally, high levels of antibodies are relatively easy to obtain in the small intestine of newborn animals by colostral transfer after vaccination of the dam. Diarrhoea caused by identical or different enterotoxigenic *E. coli* in weaned piglets is already more difficult to prevent by vaccination of the sow or of the piglets. In Belgium, very few other vaccines more specifically based upon fimbriae and adhesins are commercialised, even though they contain whole bacterins. Examples are the footrot vaccine in sheep, with the different serotypes of *Dichelobacter nodosus* and probably the vaccine against whooping-cough in humans, with extracts of *Bordetella pertussis*. The future probably resides in the use of fimbriae as epitope carriers to produce antibodies against various virulence factors.

The feasibility of using carbohydrate receptor analogs to protect against and even treat infections by bacteria

expressing lectin adhesins was experimentally demonstrated with methyl α mannoside in a mouse model about 20 years ago. This was against uropathogenic *E. coli* expressing type 1 fimbriae. The colonization of the bladder was reduced by about two-thirds in mice receiving methyl α mannoside compared to mice receiving methyl α glucoside. More recently, 50% success has been obtained in the treatment of natural *Helicobacter pylori* infections in rhesus monkeys using sialyl-glycoconjugates, which are natural receptors for some adhesins produced by this bacterial species. Good results have also been obtained in the treatment of *Pseudomonas aeruginosa* acute otitis externa by administering locally several receptors analogs in a mixture of galactose, mannose and N-acetylneuraminic acid. However these results were not as good those obtained after local administration of gentamicin !

On the contrary, the use of adhesin analogs has not received so much attention, because adhesins are macromolecules that must be employed in high concentrations and are available only in limited supply. Though the use of synthetic short peptides mimicking the adhesive domain of the adhesin molecule may prove more interesting in the future, this methodology is still at the early experimental stage.

Today, research on anti-adhesion factors focuses on the identification of receptor or adhesin analogs in dietary components, like milk, vegetables and fruit, and in probiotics, especially *Lactobacillus*. Promising results have, for example, already been obtained with cranberries. But there is still so much to do at the experimental stage, such that many years will pass before clinical applications emerge from this kind of research. Nevertheless the emergence and rapid spread of antibiotic-resistant bacteria has stimulated a growing and renewed research interest in these areas.

CONCLUSION

If we were a bacterium, we would now have successfully colonised one host epithelium and mucosa. During the next lecture, the toxins produced by pathogenic bacteria and their deleterious effects on the host cells, tissues, organs and body, will be described.

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