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ANTI-ADHESIVE THERAPIES AS A CONTEMPORARY MEANS TO FIGHT INFECTIOUS DISEASES AND ADHERENCE FACTORS OF CORYNEBACTERIA DIPHTHERIAE

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The emergence and increasing prevalence of bacterial strains that cause infectious diseases and that are resistant to available antibiotics demand the discovery of new therapeutic strategies [1]. An alternative approach to antimicrobial therapy is targeting bacterial virulence that offers promising opportunities to inhibit pathogenesis and its consequences without placing immediate life-or-death pressure on the target bacterium. Certain virulence factors have been shown to be potential targets for drug design and therapeutic intervention, whereas new insights are crucial for exploiting others. Targeting virulence represents a new paradigm to empower the clinician to prevent and treat infectious diseases [1-3].

While many antivirulence targets exist, we focuses on adhesion as an antivirulence target. If adherence can be inhibited, then the subsequent infection can also be inhibited. This approach forms the basis of anti-adherence strategies, which have been devised to prevent a variety of bacterial infections [4].

For many pathogenic bacteria, infections are initiated only after the organism has first adhered to the host cell surface. Pathogenic bacteria must stably colonize mucosal surface to initiate the infectious process [5]. Bacteria attach to their appropriate environmental niche by using adhesins. To maximize their contact with the environment, adhesins are often present on the ends of long hair-like structures called pili [6]. Adhesion to tissue surface realizes by specific interactions between molecules of recognition (receptors) on host cell surface and adhesive molecules (adhesins) on the surfaces of the bacteria [5].

With anti-adhesion therapies for bacterial infections bacteria are not killed but are prevented from causing harm to a host by inhibiting adherence to host cells and tissues [7]. There are some modern strategies for anti-adhesion therapy. For example, bacterial attachment can be inhibited by interfering with adhesin biosynthesis (1), adhesin assembly (2) or host receptor assembly (3). Binding can be inhibited by competitive replacement of the adhesin from the host (4) or of the host receptor from the adhesin (5) using soluble molecules or by using designer microbes (6). Antibodies against bacterial adhesins can block surface epitopes required for binding (7) [8].

So, the mechanisms of these potential therapeutic agents include inhibition of adhesins and their host receptors, vaccination with adhesins or analogs, use of probiotics and dietary supplements that interfere with receptor-adhesin interactions, subminimal inhibitory concentrations of antibiotics and manipulation of hydrophobic interactions [7]. The validity of this approach has been unequivocally demonstrated in experiments performed in a wide variety of animals, from mice to monkeys, and recently also in humans [9].

New efficacy *in vitro* assays have been developed allowing screening of large numbers of molecules. These inhibitors are mainly antibacterial molecules, a few natural products, peptides and antibodies. A growing number of these published studies provide results showing a proof of concept with antivirulence compounds that were able to prevent or treat an infection *in vivo*. Moreover, some new antivirulence agents could inhibit virulence mechanisms that are common to different related pathogenic species, extending the potential spectrum of antivirulence compounds. The progress reported recently for antivirulence molecules at the preclinical stages should allow new classes of molecules to enter into development as new antimicrobial agents with new mechanisms of action [10].

Among the general types of anti-adherence agents the most well-studied are the receptor analogs, which include oligosaccharides produced synthetically or derived from natural sources, including milk, berries, and other plants. Their ability to inhibit pathogen adherence may lead to development of novel, food-grade anti-infective agents that are inexpensive and safe [4].

Because anti-adhesive agents are not bactericidal, the propagation and spread of resistant strains is much less likely to occur than as a result of exposure to bactericidal agents, such as antibiotics. Anti-adhesive drugs, once developed, may, therefore, serve as a new means to fight infectious diseases [9].

Another opportunity to prevent pathogen adherence is developing and use vaccines with adhesive antigens. In the literature it's described studies on the possibility to use in therapy either purified adhesins or receptors to block receptors and ligands respectively, studies on the development of antiadhesive vaccines, composed of purified ligands, to elicit local immune responses, resulting in the secretion of antibodies that cover the bacterial adhesins and thereby prevent attachment [5]. Also they have reported about the development and use of antibodies or antisera directed against bacterial adhesins as an anti-adhesive strategy [8, 11]. The host can be directly or passively immunized using a bacterial adhesin, an adhesin subunit (as in the case of multi-subunit adhesive organelles, such as fimbriae), or an immunogenic peptide fragment based either on an individual adhesin or on the consensus derived from a group of adhesins. Lastly, the host can be immunized using a DNA vaccine encoding the adhesin or part thereof [8].

Subunit-based vaccines are often used in the context of fimbrial adhesins and several of them have been described for therapeutic use against pathogenic *E. coli.* Enterotoxigenic *E. coli* (ETEC) are a major cause of

diarrheal disease in humans and other animals and pathogenicity is to a large extent caused by enterotoxins. A fusion protein consisting of FaeG, the major subunit of *E. coli* K88ac fimbriae, an epitope from the B subunit of heat-labile (LT) toxin and the A subunit of shiga toxin (STa) was used to immunize rabbits. Data from this study demonstrated that K88ac fimbriae expressing LT and STa epitope antigens elicited neutralizing anti-toxin antibodies and anti-adhesin antibodies and suggested that *E. coli* fimbriae could serve as a platform for the development of broad-spectrum vaccines against ETEC. Anti-K88ac, anti-LT and anti-STa antibodies inhibited adhesion of fimbrial *E. coli* to small intestinal enterocytes and neutralized both shiga toxin and cholera toxin [8, 12].

A similar strategy was pursued to generate antibodies against enterohemorrhagic *E. coli* (EHEC). EHEC attachment to the host is primarily due to intimin and the subsequent pathology is caused by shiga toxin. A SSI (Statens Serum Institute) fusion protein containing two different toxin antigens as well as an intimin antigen fragment, was used to immunize mice. The fusion protein induced a strong humoral immune response, with both toxin neutralizing and anti-adhesion antibodies being generated. Subsequent bacterial challenge of twice-immunized mice with an otherwise lethal dose of EHEC strain O157:H7 did not cause any pathology [8, 13]. SSI protein provides evident protection with two-time immunization against a highly lethal dose of *E. coli* O157:H7.

The use of preventive vaccines against recurring urinary tract infections (UTIs) based on fimbrial subunits FimCH has been investigated quite extensively both in animal models and clinical trials. Immunization with candidate FimH vaccines reduced *in vivo* colonization of the bladder mucosa by more than 99 % in a murine model [8, 11, 14].

A major focus in the development of vaccines and in antibody-based therapeutic strategies is inhibiting antibodies targeting receptor-binding pockets in proteins. By using a common mannose-specific fimbrial adhesin of *Escherichia coli*, FimH, it's demonstrated that locking the adhesin in a low-binding conformation induces the production of binding pocket-specific, adhesion-inhibiting antibodies [11]. A di-sulfide bridge was introduced into the conformationally dynamic FimH lectin domain, away from the mannose-binding pocket but rendering it defective with regard to mannose binding. Unlike the native, functionally active lectin domain, the functionally defective domain was potent in inducing inhibitory monoclonal antibodies that blocked FimH-mediated bacterial adhesion to epithelial cells and urinary bladder infection in mice. Inhibition of adhesion involved direct competition between the antibodies and mannose for the binding pocket. Binding pocket-specific inhibitory antibodies also were abundant in polyclonal immune serum raised against the functionally defective lectin domain. The monoclonal antibodies elicited against the binding-defective protein bound to the high-affinity conformation of the adhesin more avidly than to the low-affinity form. However, both soluble mannose and blood plasma more strongly inhibited antibody recognition of the high-affinity FimH conformation than the low-affinity form. Authors hypothesized that in the functionally active conformation the binding-pocket epitopes are shielded from targeted antibody development by ligand masking and that strong immunogenicity of the binding pocket is unblocked when the adhesive domain is in the nonbinding conformation [11].

Immunization of monkeys with FimCH adhesin–chaperone complex in combination with an adjuvant elicited a strong IgG antibody response and protected 3/4 of the animals against subsequent uropathogenic *E. coli* (UPEC) infection [8, 15]. These findings suggest that a vaccine based on the FimH adhesin of *E. coli* type 1 pili may have utility in preventing cystitis in humans. Urinary tract infections (UTIs) are considered the most common bacterial infections, especially in women [8, 16].

The treatment for *Salmonella enterica serovar Typhi*, the causative agent of typhoid fever, is increasingly complicated due to the emergence of multidrug resistant strains. The *S. typhi* adhesin T2544 is a major contributor to bacterial host interaction and disease pathogenesis and a potential target for development of an anti-adhesion vaccine. Deletion of T2544 results in reduced systemic invasion and a 10-fold increase in LD_{50} in a murine model. T2544 is highly immunogenic and elicits elevated titers of serum IgG and intestinal secretory IgA in immunized mice. T2544 antiserum enhances both the uptake and clearance of bacteria by macrophages as well as complement-mediated lysis. Mice either immunized with T2544 or passively immunized with anti-T2544 antiserum were protected against subsequent bacterial challenge and showed increased bacterial shedding [8, 15]. However, T2455-based immunization did not completely inhibit disease and this is likely due to other structures promoting cellular invasion, such as type IV pili [8, 17-19].

With *S. typhimurium*, the effect of multifactorial adhesion on the outcome of immunization attempts is equally problematic. SadA, a purported trimeric autotransporter adhesin of *Salmonella enterica* serovar *Typhimurium* involved in cell aggregation, biofilm formation, autoaggregation and host binding by *S. typhimurium*, was tested as a vaccine candidate. Expression of SadA resulted in increased adhesion to human intestinal Caco-2 epithelial cells. Although purified SadA itself triggered an immune response, which was even more pronounced when administered together with an adjuvant, it provided only limited protection against subsequent bacterial challenge [8, 20]. As it's turned out it's not a dominant, protective antigen for antibody-mediated protection against Salmonella.

A cheap and efficient way to achieve the necessary multivalency of inhibitive epitopes is their heterologous expression on the surface of probiotic bacteria. The protective effects of probiotic bacteria against infections has long been appreciated, and has been systematically demonstrated in a range of trials [8].

Probiotic strains have also been used to reduce pathogen colonization of animals raised for human consumption. For example, treatment of broiler chicken with a multi-species probiotic consisting of bacteria isolated from the chicken gut prevented their colonization by *Campylobacter jejuni*. The beneficial effects of probiotics are, to some extent, due to competitive exclusion of pathogenic bacteria from host binding sites although this is challenging to demonstrate in vivo because of the complexity of the probiotics' mechanisms of action. Over recent years, probiotics have been specifically engineered to mimic sugars on host receptors, thereby blocking the host cell binding of toxins released by pathogenic bacteria including ETEC, shiga toxin-producing *E. coli* (STEC) and *V. cholerae*. [8, 12].

Pseudomonas aeruginosa and *Pseudomonas maltophilia* account for 80 % of opportunistic infections by pseudomonads. *Pseudomonas aeruginosa* is an opportunistic pathogen that causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, and a variety of systemic infections, particularly in patients with severe burns, and in cancer and AIDS patients who are immunosuppressed. *Pseudomonas aeruginosa* is notable for its resistance to antibiotics, and is therefore a particularly dangerous pathogen. The identification and isolation of a small peptide structural element found in *P. aeruginosa* strain K (PAK) bacterial pili, which has been proven to function as a host epithelial cell-surface receptor binding domain were described in the study [8, 21]. Several of heterologous peptide founded in the pili of all strains of *P. aeruginosa* sequences have been used in the development of an consensus sequence anti-adhesin vaccine targeted at the prevention of host cell attachment and further for the generation of a monoclonal antibody capable of prevention and treatment of existing infections [8, 21].

Another study utilizing *P. aeruginosa* strain K pili as an immunogenic target described the generation of monoclonal antibodies which were also cross-protective against *P. aeruginosa* strain O (PAO) and *P. aeruginosa* strain K (PAK) when used for active immunization in a murine model. The authors noted that the generation of an effective antibody relied on the appropriate presentation of the immunogenic peptide, which was achieved through conformational restriction of the peptide by both C- and N-terminal coupling to a carrier [8, 22].

To improve surface display of bacterial antigens and trigger both humoral and cellular immune responses, several approaches have been taken to ensure the epitope is displayed in a physiological conformation. For example, this can be achieved by associating adhesin antigens with outer membrane vesicles and this strategy was used to create vaccines against *Neisseria meningitides*, a causative agent of meningitis and septicemia [8, 23-25].

Acellular pertussis vaccines can be called as one of the modern examples of use of adhesins in immuno-prophylactic preparations [26-30, 31-37]. The main component of many pertussis acellular vaccines is pertussis toxoid. But if the vaccine contains only pertussis toxoid, it prevents only severe pertussis which accompanies with spasmodic cough with a period of over 3 weeks. To prevent mild forms of the disease which is particularly important for the family members of the patient, as well as during epidemics adhesins (pertactin, fimbriae) and filamentous hemagglutinin should be included in the vaccine.

Pertactin is an outer membrane protein with a molecular weight of 69 000 kDa. It's a factor that mediates the close relationship between the bacterium *Bordetella pertussis* and macroorganism cells. Filamentous hemagglutinin (FHA) is an another factor that mediates that relationship and the second component of many pertussis acellular vaccines. Rod-shaped molecules of this surface protein have a molecular weight of 220 000 kDa. There are an incorporated repeating sequence arginine-glycine-aspartic acid in pertactin and filamentous hemagglutinin, which makes them similar to integrins belonging to a family of adhesion molecules in mammals. Antibodies are produced by first administering of the FHA antigen, prevent adhesion pertussis pathogen, however, if it does occurs, the main component antibody neutralizes pertussis toxin which defines clinical symptoms of the disease generally.

The next colonization factor antigens of *B.pertussis* are fimbriae (pili). They are threadlike protein organelles that penetrate the outer membrane and cover the entire surface of the pertussis bacterial cell. The separate cells may have several hundred fimbriae that perform different functions. It's believed that ensuring fixing of bacteria in tissues is the main function of pili. Pili are composed of a plurality of identical protein subunits. This subunit called pilin, its molecular weight is from 17 000 to 30 000 kDa. Conserved and variable regions are included in pilin. Chromosome rearrangements leading to the expression of any of the plurality of inactive pilin genes, accompanied by changes in the antigenic composition of pili [26-30, 31-37].

Antigens can be displayed on the surface of live, attenuated bacterial strains which can be used as oral vaccines [8, 12, 14, 38]. A recent clinical study of sublingual bacterial vaccine Uromune® which contained an inactivated bacterial cell suspension of selected strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Enterococcus faecalis* comparing the efficacy of preventive vaccination to prophylactic treatment with antibiotics concluded that vaccination was a more effective strategy to reduce frequency, duration and severity of recurring UTIs [8, 12].

This is a low cost approach and thus particularly suitable for the prevention of zoonotic infectious diseases as it could be used to immunize large herds of animals. For example, a live attenuated *S.typhimurium* strain expressing a combination of *E. coli* fimbrial antigens (K88ab, K88ac, FedA, and FedF) prevented post-weaning diarrhea in piglets when used to immunize pregnant pigs [38]. No environmental exposure was reported because no live bacteria from the vaccine strain

were shed by the animals following immunization [8, 38, 39].

Vaccination with recombinant adhesins from the RgpA-Kgp proteinase-adhesin complex protects against *Porphyromonas gingivalis* infection. All of the recombinant adhesin domains were found to significantly attenuate *P. gingivalis* infection [40-42]. The *P. gingivalis* cell surface RgpA-Kgp proteinase-adhesin complex and synthetic ABM (potential adhesin binding motifs) and proteinase active site peptides conjugated to diphtheria toxoid, when used as vaccines, protected against *P. gingivalis*-induced periodontal bone loss in the murine periodontitis model. The results suggest that when the RgpA-Kgp complex, or functional binding motif or active site peptides are used as a vaccine, they induce a Th2 response that blocks function of the RgpA-Kgp complex and protects against periodontal bone loss [42]. The recombinant A1 adhesin therefore has potential in the development of a *P. gingivalis* vaccine [41, 42]. The Triton-wt complex induced a stronger antibody response to the A1 adhesins and tended to be more effective in providing protection in the mouse lesion model compared with the sonication-wt complex [42].

DNA vaccines contain DNA encoding pathogen-derived antigens, which upon their expression in the host are able to elicit protective immunity. Theoretically, this strategy is advantageous because it improves antigen processing and presentation and induces both humoral and cellular immune responses [8, 43-46]. DNA vaccines have been generated and tested as a tool to prevent *S. aureus* infections. Treatment of staphylococcal infections becomes increasingly complicated by the high level of multidrug resistance seen with *S. aureus. S. aureus* binding to host cells is mediated by a number of surface proteins binding to extracellular matrix components with extremely high affinity. A DNA vaccine based on collagen-binding protein (CNA), a major *S. aureus* adhesin, was used to immunize Balb/c mice. Mice injected with three doses of the eukaryotic expression vector pCNA, expressing the collagen-binding domain of CNA, showed evidence of both antibody- and cell-mediated immune response against CNA. Even though the antibodies recognized intact bacteria and inhibited binding to collagen *in vitro*, they failed to protect mice against intra-peritoneal infection by *S. aureus* [8, 45]. A polyprotein DNA vaccine against *S. aureus*, consisting of a series of plasmids expressing clumping factor A (ClfA), fibronectin binding protein A (FnBPA), and the enzyme sortase (Srt), triggered both antibody production and T-cell response and provided partial protection against *S. aureus* isolate Sa042 and full protection against reactive arthritis after challenge with *S. aureus* strain Newman [8, 47].

One of the major drawbacks of anti-adhesion therapy is the high degree of redundancy in bacterial adhesive strategies that in many cases interfere with effective treatment. The use of anti-adhesion antibodies or vaccines may still be effective in such cases as antibody opsonization can increase bacterial uptake and clearance by macrophages and antibodies may trigger complement-mediated bacteriolysis, even if they are unable to fully inhibit bacterial adhesion [15]. Although one could argue that antigenic variability of bacterial adhesins can potentially impair the efficacy of anti-adhesion antibodies, the fact is that many adhesins show a remarkable degree of conservation, making them good vaccine candidates [8, 9, 48].

An intensive field of investigation and pinpoints the areas that need further study are pili [49]. Bacterial pili are defined as non-flagellar, proteinaceous, multi-subunit surface appendages involved in adhesion to other bacteria, host cells, or environmental surfaces [50-52]. Pili were first recognized by electron microscopy on Gram-negative bacteria more than 50 years ago [50, 53] and on Gram-positive bacteria more than 40 years ago [50, 54].

Bacterial pili have long been recognized as mediators of initial host-pathogen interactions important for the progression of Gram-negative bacterial diseases [55]. Pili of Gram-negative pathogens are formed from pilin precursor molecules by non-covalent association within the outer membrane envelope [56]. It's reported about two strategies of drug development involved the inhibition of bacterial binding to the host tissue by the addition of exogenous sugars, and the disruption of chaperone-usher (CU) pilus assembly through the disruption of protein-protein interactions. They used pili of uropathogenic *E. coli* as a model system and believed that because of the commonality of adhesion to infectious disease processes and CU pili to many pathogens, the two strategies are translatable to a multitude of organisms [1].

The chaperone-usher (CU) pathway of pilus biogenesis is the most widespread of the five pathways that assemble adhesive pili at the surface of Gram-negative bacteria. Recent progress in the study of the structural biology of the CU pathway has unravelled the molecular basis of chaperone function and elucidated the mechanisms of fibre assembly at the outer membrane, leading to a comprehensive description of each step in the biogenesis pathway. Other studies have provided the molecular basis of host recognition by CU pili. The knowledge that has been gathered about both the assembly of and host recognition by CU pili has been harnessed to design promising antibiotic compounds [57].

Recently, attention has focused on pili of Gram-positive bacteria because they may be vaccine candidates in important human pathogens. Pili are highly immunogenic structures that are under the selective pressure of host immune responses [49]. Pili of Gram-positive bacteria have not been extensively investigated yet [6]. Since the beginning of their study time it's ascertained that cell surface pili in gram-positive bacteria have been implicated in critical host-pathogen interactions, in adhesion to host extracellular matrix proteins, colonization of host tissues, tissue tropism determination, biofilm formation as one of mechanisms of persistence in human organism, signaling events, modulation of innate immune responses and their contribution to virulence, and the regulation of their

expression, a characteristic life-style of the bacteria constituting the oral flora and so on [49, 50, 58-65]. Gram-positive pathogens use a variety of surface proteins as a key virulence factors of most bacterial pathogens to bind to host tissues, evade the innate and acquired immune systems, and invade host epithelial and immune cells [58-60]. Gram-positive microbes employ the cell wall peptidoglycan as a surface organelle for the covalent attachment of proteins [56].

In the last decade, pili, which are encoded within pathogenicity islands, have been found in many Gram-positive bacteria, including the major streptococcal and enterococcal pathogens. These long proteinaceous polymers extending from the bacterial surface are constituted of covalently linked pilin subunits, which play major roles in adhesion and host colonization [49].

Indeed, pilus expression was found to be heterogeneous in several bacteria with the co-existence of two subpopulations expressing various levels of pili. The molecular mechanisms underlying this complex regulation are poorly characterized except for *Streptococcus pneumonia* [49].

Gram-positive microbes employ the cell wall peptidoglycan as a surface organelle for the covalent attachment of proteins, however, an assembly pathway for pili has not yet been revealed [56]. An appreciation of the role of pili on virulence in Gram-positive bacteria and the unique properties of their biogenesis is a rapidly emerging area of research [55].

These pili differ from the well-studied pili of Gram-negative bacteria because their subunits are covalently linked, they do not require specific chaperones for assembly, and the tip protein (likely to be the adhesin) is not required to initiate formation of the pilus structure. In Gram-positive bacteria, the genes for pili occur in clusters, which may constitute mobile genetic elements. These clusters include the transpeptidase(s) of the sortase family that is/are required for polymerization of the subunit proteins. However, efficient covalent attachment of the completed pilus structure to the cell wall is accomplished, in cases where this has been studied, by the 'housekeeping' sortase, which is responsible for attachment to the peptidoglycan of most surface proteins containing cell wall sorting signals. This enzyme is encoded elsewhere on the genome [6].

We'll dwell on adherence factors of *Corynebacteria* as probable components of developing diphtheria vaccines. Pathogenic members of the genus *Corynebacterium* cause a wide range of serious infections in humans including diphtheria. The epidemic re-emergence of diphtheria in Russia and the Newly Independent States of the former Soviet Union in the last decade of the past century demonstrated the continued threat of this thought to be rare disease [60]. Diphtheria epidemic in countries of Eastern Europe in the 1990s testifies preservation of the reservoir of infection in modern society [60, 66-69]. *Corynebacterium diphtheriae* still represents a global medical challenge, particularly due to the significant number of individuals susceptible to diphtheria and the emergence of non-toxigenic strains as the causative agents of invasive infections [61, 70].

A main virulence factor of *C. diphtheriae* is the bacteriophage encoded toxin, however, an analysis of the first complete genome sequence of *C. diphtheriae* revealed a recent acquisition of other pathogenicity factors including iron-uptake systems, adhesins and fimbrial proteins as indeed this extracellular pathogen has more possibilities for lateral gene transfer than, e.g., its close relative, mainly intracellular *Mycobacterium tuberculosis*. *C. diphtheriae* appears to have a phylogeographical structure mainly represented by area-specific variants whose circulation is under strong influence of human host factors, including health control measures, first of all, vaccination, and social economic conditions. This framework core population structure may be challenged by importation of the endemic and eventually toxigenic strains from new areas thus leading to localized or large epidemics caused directly by imported strains or by bacteriophage-lysogenized indigenous strains converted into toxin production. A feature of *C. diphtheriae* co-existence with humans is its periodicity: following large epidemic in the 1990s, the present period is marked by increasing heterogeneity of the circulating populations whereas re-emergence of new toxigenic variants along with persistent circulation of invasive non-toxigenic strains appear alarming [60].

The characterized strains *C. diphtheriae* on the basis of the chosen criteria namely adhesive, invasive and cytotoxic activity could be subdivided into highly, moderately and low virulent and the degree of their potential epidemic danger could be determined [71]. Among non-toxigenic strains those with intermediate adhesiveness were predominated (45,5 %) [72]. The study of the main pathogenicity factors of *C. diphtheriae* (adhesive activity, toxigenicity, detection of tox+ gene) circulating in the Primorski Territory (Russia) has been revealed that at the period of declined epidemic process the selection of *C. diphtheriae* strains with weak toxigenicity and low adhesiveness was observed [73].

C. diphtheriae, the causative agent of diphtheria, is well-investigated in respect to toxin production, while little is known about *C. diphtheriae* factors crucial for colonization of the host [74]. It's known that anti-toxic immunity doesn't interfere with a carriage of toxigenic *C. diphtheria* strains, their invasion and persistence in a human body [61, 62]. A factor which localizes a diphtheritic infection in an organism is an antibacterial immunity. It's characterized by cell-mediated immunity and delayed-type hypersensitivity. The carriage is formed against the background of the reduced antibacterial antidiphtheritic immunity and defines preservation of the reservoir of infection in human population [75]. Effective methods of treatment of a resistant carrier state are absent. Experimental studies proved need of search of the bacterial antigenes responsible for formation of organism resistance to an invasion of the pathogen [76]. Adhesion to host cells is a crucial step during infection [77].

So, although diphtheria has been studied to a great extent, relatively little is known still about the two key aspects of *C. diphtheriae* invasiveness: colonization and invasion. The role of adhesive properties in establishing the infection of *C. diphtheriae* strains, independent of toxin production, still needs to be clarified [74, 78]. It's necessary to investigate various isolates on a molecular level to understand and to predict the colonization process of different *C. diphtheriae* strains [74].

In *C. diphtheriae*, adhesion is mediated primarily by filamentous structures called pili (or fimbriae) that are covalently attached to the bacterial cell wall [58, 59, 77]. Wild type *C. diphtheriae* cells were shown to bind to human lung epithelial, laryngeal, and pharyngeal cells [58]. The first observation that corynebacterial pili may mediate host cell interactions was proposed in early studies with *C. renale* pili, which were shown to agglutinate trypsinized sheep red blood cells [58, 59, 79]. Using ultrastructure analyses by atomic force microscopy, significant differences in macromolecular surface structures were found between the investigated *C. diphtheriae* strains in respect to number and length of pili [74]. Three distinct pilus structures can be produced by *C. diphtheriae*: SpaA-, SpaD- and SpaH-type pili [77, 80]. Similar to other types, the prototype SpaA pilus consists of SpaA, the major pilin protein, forming the pilus shaft and two minor pilins SpaB and SpaC located at the base and at the tip, respectively [50, 56, 63, 77, 80, 81]. SpaA is distributed uniformly along the pilus shaft, whereas SpaB is observed at regular intervals, and SpaC seems to be positioned at the pilus tip [82]. For the first time Mandlik et al. identified the minor pilins SpaB and SpaC of *C. diphtheria* as specific adhesins that mediate efficient corynebacterial adherence to host pharyngeal cells [58].

Pilus genes encode a total of nine pilus proteins, named SpaA through SpaI, and six sortases, named SrtA through SrtF, five of which are devoted to the assembly of three distinct types of pilus fibres – SrtA for the SpaA-type pilus, SrtB/SrtC for the SpaD-type pilus, and SrtD/SrtE for the SpaH-type pilus [81]. The genome of *C. diphtheria* NCTC13129 contains three pilus gene clusters, which encode for the SpaA-type pili (*spaA-srtA-spaB-spaC*), the SpaD-type pili (*srtB-spaD-srtC-spaE-spaF*) and the SpaH-type pili (*spaG-spaH-srtD-srtE-spaI*). [82]. All the pilus genes are located on pathogenicity islands and can be acquired and lost by different strains [80]. The housekeeping sortase, *srtF*, is located elsewhere on the chromosome [82]. Similar pilus gene clusters and the housekeeping sortase gene are found in many pathogenic species of the genus *Corynebacterium* [82].

Invasiveness of *C. diphtheriae* strains could be related to adhesive factors. Most probably ability to express all types of pili is indispensable for causing invasive infections by nontoxigenic *C. diphtheriae*. Whereas full set of pili genes is not necessary for causing classical diphtheria [80].

It's described a novel *C. diphtheriae* gene involved in adherence to epithelial cells. Transformation of *C. diphtheriae* 225, biotype gravis, ribotype St-Petersburg by EZ:TN(KAN-2)Tnp Transposome was undertaken. A *C. diphtheriae* 225 Tn5 insertion library of 2800 mutants was created. Five hundred and eighty five transformants were qualitatively screened for reduced adherence to HEp-2 cells by an adherence assay. One mutant strain consistently exhibiting 15.2 % of the wild-type adherence was isolated. The DNA flanking the transposon was identified by inverse PCR and subsequent sequencing. The disrupted gene was 94 percent identical to the *C. diphtheriae* DIP1621 gene that belongs to unclassified genes. The disruption of the *C. diphtheriae* DIP1621 gene led to decreased adherence to epithelial cells [78].

Importantly, the host cell receptor(s) targeted by SpaB and SpaC have yet to be identified as well as the specific functions of the SpaD- and SpaH-type pili. Such studies are likely to be rewarding because some important corynebacterial pathogens do not contain the SpaA-type pilus but harbor only the SpaD-type or the SpaH-type pilus [58].

Like pili of many other gram-positive microbes, the assembly of corynebacterial pili occurs by a two-step mechanism, whereby pilins are covalently polymerized by a transpeptidase enzyme, pilin-specific sortase, and the generated pilus polymer is subsequently anchored to the cell wall peptidoglycan via the base pilin by the housekeeping sortase SrtF or a non-polymerizing sortase [50, 63, 77, 81]. In *C. diphtheriae*, the pilin-specific sortase SrtA catalyses polymerization of the SpaA-type pilus, consisting of the shaft pilin SpaA, tip pilin SpaC and minor pilin SpaB [50].

Recent work revealed that a multiple deletion mutant strain expressing only SrtA secretes a large portion of SpaA polymers into the culture medium, with concomitant decrease in the cell wall-linked pili. The same phenotype is observed with the mutant that is missing SrtF alone. By contrast, a strain that expresses only SrtF displays surface-linked pilins but no polymers. Therefore, SrtF can catalyse the cell wall anchoring of pilin monomers as well as pili, but it does not polymerize pilins. SrtA and SrtF together generate wild-type levels of the SpaA-type pilus on the bacterial surface. Furthermore, by regulating the expression of SpaA in the cell, the SrtF function becomes critical when the SpaA level is sufficiently high. Thus, these findings provide key evidence for a two-stage model of pilus assembly: pilins are first polymerized by a pilus-specific sortase, and the resulting fibre is then attached to the cell wall by either the cognate sortase or the housekeeping sortase [81]. Cell wall anchoring of the SpaA polymers is triggered when SrtA incorporates SpaB into the pilus base via lysine-mediated transpeptidation; anchoring to the cell wall peptidoglycan is subsequently catalysed by the housekeeping sortase SrtF [50, 63].

SpaB and SpaC formed a heterodimer independent of SpaA polymerization. SrtA was absolutely required for the formation of the SpaBC heterodimer, while SrtF facilitated the optimal cell wall

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anchoring of this heterodimer. Alanine substitution of the SpaB lysine residue K139 or truncation of the SpaB cell wall-sorting signal (CWSS) abolished assembly of the SpaBC heterodimer, hence underscoring SpaB function in transpeptidation and cell wall linkage. Importantly, sortase specificity for the cell wall-anchoring step was found to be dependent on the LAFTG motif within the SpaB CWSS. Thus, *C. diphtheriae* employs a common sortase-catalysed mechanism involving lysine-mediated transpeptidation to generate both adhesive pilus and simple heterodimeric structures on the bacterial cell wall [50].

Cells lacking SpaB form pilus fibers, but they are largely secreted in the medium, a phenotype also observed when cells lack the housekeeping sortase. Furthermore, the average pilus length is greatly increased in the absence of SpaB. Remarkably, a SpaB mutant that lacks the cell wall sorting signal but contains a critical lysine residue is incorporated in the pilus. However, the resulting pili fail to anchor to the cell wall. Probably a specific minor pilin acts as the terminal subunit in pilus assembly. Cell wall anchoring ensues when the pilus polymer assembled on the pilus-specific sortase is transferred to the minor pilin presented by the housekeeping sortase via lysine-mediated transpeptidation [63]. The minor pilins SpaB/SpaC are critical for bacterial binding to human pharyngeal cells, and thus represent the major adhesins of corynebacteria [77].

Besides that as it's reported mutants that lacked SrtA (and thus did not polymerize SpaA-type pili) showed over a 90 % reduction in the ability to adhere to human pharyngeal cells. Surprisingly, mutants that lacked only the major pilin subunit, SpaA, showed a modest 10 % reduction in adherence to these cells. This is in contrast to mutants that lacked either of the minor pilin subunits, SpaB or SpaC, which showed a 70–75 % reduction in adherence. Additionally, latex beads coated with only SpaB or SpaC were sufficient to adhere to host pharyngeal cells, while beads coated with SpaA showed no binding. As mentioned above, SpaB and SpaC anchor to the cell wall as monomers independent of pilus structures. It is speculated that the long pili mediate initial attachment, whereas monomeric pilins on the bacterial surface may help to create an intimate zone of adhesion that allows for efficient delivery of toxin and other virulence factors, and may even play a significant role in host cell signaling [77, 58].

Assembled pili are released from the bacterial surface by treatment with murein hydrolase. All three pilin subunit proteins are synthesized as precursors carrying N-terminal signal peptides and C-terminal sorting signals. Some, but not all, of the six sortase genes encoded in the genome of *C. diphtheriae* are required for precursor processing, pilus assembly or cell wall envelope attachment [56]. Pilus assembly in *C. diphtheriae* requires the pilin motif and the C-terminal sorting signal of SpaA, and is proposed to occur by a mechanism of ordered cross-linking, whereby pilin-specific sortase enzymes cleave precursor proteins at sorting signals and involve the side-chain amino groups of pilin motif sequences to generate covalent linkages between pilin subunits. This covalent tethering of adjacent pilin subunits appears to have evolved in many Gram-positive pathogens that encode sortase and pilin subunit genes with sorting signals and pilin motifs [56, 82].

Two elements of SpaA pilin precursor, the pilin motif and the sorting signal, are together sufficient to promote the polymerization of an otherwise secreted protein by a process requiring the function of the sortase A gene (srtA). Five other sortase genes are dispensable for SpaA pilus assembly. Further, the incorporation of SpaB into SpaA pili requires a glutamic acid residue within the E box motif of SpaA, a feature that is found to be conserved in other Gram-positive pathogens that encode sortase and pilin subunit genes with sorting signals and pilin motifs. When the main fimbrial subunit of *Actinomyces naeslundii* type I fimbriae, FimA, is expressed in corynebacteria, *C. diphtheriae* strain NCTC13129 polymerized FimA to form short fibres. Although *C. diphtheriae* does not depend on other actinomycetal genes for FimA polymerization, this process involves the pilin motif and the sorting signal of FimA as well as corynebacterial sortase D (SrtD). Thus, pilus assembly in Gram-positive bacteria seems to occur by a universal mechanism of ordered cross-linking of precursor proteins, the multiple conserved features of which are recognized by designated sortase enzymes [56, 82].

Many studies have examined specific surface components of *C. diphtheriae* that may be involved in adhesion [58]. Among of them is a lipoglycan called CdiLAM. The key structural features of CdiLAM are a linear alpha-1-->6-mannan with side chains containing 2-linked alpha-D-Manp and 4-linked alpha-D-Araf residues. (LAM – lipoarabinomanan). The polysaccharide backbone is linked to a phosphatidylinositol anchor. Unlike the non-fimbrial adhesin 62-72p, CdiLAM did not function as a hemagglutinin to human red blood cells. Experimental evidences pointed to CdiLAM as an adhesin of *C. diphtheriae* to human respiratory epithelial cells. Preincubation of bacteria with anti-CdiLAM significantly inhibited adherence to HEp-2 cells [58, 83].

Another one identified in *C. diphtheriae* is a cell surface protein, DIP1281. Mutants lacking DIP1281 show over 3-fold reduction in adherence to human pharyngeal cells compared to their isogenic parental strains [58]. Importantly, the DIP1281 mutant was also defective in invasion.

Subinhibitory concentrations (subMICs) of antibiotics may alter bacterial surface properties and change microbial physiology. Adherence to human erythrocytes was reduced after growth in the presence of erythromycin. All strains enhanced biofilm formation on glass after treatment with erythromycin. Antibiotic-induced biofilm formation may contribute to the inconsistent success of antimicrobial therapy for *C. diphtheriae* infections [84]. It has been established that only erythromycin in minimum inhibiting concentration manifested the ability to inhibit the adhesion of different

biovars of *C. diphtheriae*. The drug action did not depend on the level of adhesive activity of certain cultures [85].

As ascertained adhesion and pili formation are not coupled processes and also no correlation between invasion and pili formation was found in different *C. diphtheriae* strains, that is adhesion to epithelial cells and invasion of these cells are not strictly coupled processes. Using RNA hybridization and Western blotting experiments, strain-specific pili expression patterns were observed. None of the studied *C. diphtheriae* strains had a dramatic detrimental effect on host cell viability as indicated by measurements of transepithelial resistance of Detroit 562 cell monolayers and fluorescence microscopy, leading to the assumption that *C. diphtheriae* strains might use epithelial cells as an environmental niche supplying protection against antibodies and macrophages [74].

It is obvious that antitoxic immunity does not prevent carriage of *C. diphtheria*, its colonization of mucous membrane and stay in the human body. Lack of effective treatments for toxigenic Corynebacterium carriers, especially long-term, results in the preservation of the reservoir of diphtheriae infection among human population. Investigation of pili of Gram-positive bacteria is the area most in need of further study. Development of a new class of combined diphtheria candidate vaccines with an anti-adhesion action can be an effective means of the limiting of mucous membranes colonization processes and reduce the circulation of *C. diphtheria* among population.

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ANTI-ADHESIVE THERAPIES AS A CONTEMPORARY MEANS TO FIGHT INFECTIOUS DISEASES AND ADHERENCE FACTORS OF CORYNEBACTERIA DIPHTHERIAE

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The emergence and increasing prevalence of bacterial strains that cause infectious diseases and that are resistant to available antibiotics demand the discovery of new therapeutic strategies. For many pathogenic bacteria, infections are initiated only after the organism has first adhered to the host cell surface. A modern alternative approach to antimicrobial therapy is targeting bacterial virulence and specifically adhesion as one of virulence factors. This approach forms the basis of anti-adherence strategies, which have been devised to prevent a variety of bacterial infections. The article deals with some modern strategies for anti-adhesion therapy, the mechanisms of inhibition pathogen adherence, immunization using a bacterial adhesion, an adhesin subunit or an immunogenic peptide fragment and a DNA vaccine encoding the adhesin or part thereof and so on. Investigation of bacterial pili which orchestrate the colonization of host tissues is the area most in need of further study. Bacterial pili may be vaccine candidates in important human pathogens as being highly immunogenic structures which are under the selective pressure of host immune responses. *C. diphtheriae*, the causative agent of diphtheria, is well-investigated in respect to toxin production, while little is known about its factors crucial for colonization of the host. Adherence factors of *Corynebacteria* may be considered as probable components of developing of combined diphtheria vaccines with antibacterial action which localizes a diphtheritic infection in an organism. They'll become an effective method against a resistant carrier state and be forward to stopping of pathogen circulation among human population.

Key words: ***a***dhesion, pili, *C. diphtheriae*, anti-adhesion therapy, antibacterial immunity, diphtheria vaccines

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АНТИАДГЕЗИВНА ТЕРАПІЯ ЯК СУЧАСНИЙ ЗАСІБ БОРОТЬБИ З ІНФЕКЦІЙНИМИ ХВОРОБАМИ ТА АДГЕЗИВНІ ФАКТОРИ КОРИНЕБАКТЕРІЙ ДИФТЕРІЇ

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Виникнення і розповсюдження бактеріальних штамів, котрі викликають інфекційні захворювння і є стійкими до антибіотиків, потребують відкриття нових терапевтичних стратегій. Для багатьох патогенних бактерій інфекційний процесс ініціалізується лише після адгезії мікроорганізму на

поверхні клітин організму хазяїна. Сучасним альтернативним підходом до антимікробної терапії є націлювання на бактерійну вірулентність та, особливо, адгезію як один з факторів вірулентності. Цей підхід формує основу антиадгезивних напрямків лікування, розроблених для попередження багатьох бактерійних інфекцій. У статті йдеться про деякі сучасні стратегії антиадгезивної терапії, механізми пригнічення адгезії патогену, імунізацію з використанням бактерійних адгезинів, адгезивних субодиниць або фрагментів імуногенних пептидів і ДНК вакцин, кодуючих адгезин або його частину, і т.і. Дослідження бактеріальних пілей, котрі керують колонізацією тканин хазяїна, є областю, яка нагально потребує подальшого вивчення. Бактерійні пілі, котрі є високо імуногенними структурами, знаходяться під селективним тиском імунної відповіді хазяїна і можуть бути кандидатами у вакцини для багатьох важливих патогенів людини. *C. diphtheriae*, збудник дифтерійної інфекції, добре вивчений у відношенні токсиноутворення, тоді як про його фактори, вирішальні для колонізації хазяїна, відомо мало. Адгезивні фактори *Corynebacteria* можна роглядати як вірогідні компоненти комбінованих дифтерійних вакцин з антибактерійною дією, котрі локалізують дифтерійну інфекцію в організмі. Вони можуть стати ефективним засобом проти стійкого бактеріоносійства і будуть сприяти припиненню циркуляції збудника в людській популяції.
Ключові слова: адгезія, пілі, *C. diphtheriae*, анти-адгезивна терапія, антибактерійний імунитет, дифтерийні вакцины

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АНТИАДГЕЗИВНАЯ ТЕРАПИЯ КАК СОВРЕМЕННЫЙ СПОСОБ БОРЬБЫ С ИНФЕКЦИОННЫМИ ЗАБОЛЕВАНИЯМИ И АДГЕЗИВНЫЕ ФАКТОРЫ КОРИНЕБАКТЕРИЙ ДИФТЕРИИ

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Возникновение и распространение бактериальных штаммов, которые вызывают инфекционные заболевания и являются устойчивыми к антибиотикам, требуют открытия новых терапевтических стратегий. Для многих патогенных бактерий инфекционный процесс инициализируется только после адгезии микроорганизма на поверхности клеток организма хозяина. Современным альтернативным подходом к антимикробной терапии является нацеливание на бактериальную вирулентность и, особенно, адгезию как один из факторов вирулентности. Этот подход формирует основу антиадгезивных направлений лечения, разработанных для предупреждения множества бактериальных инфекций. В статье речь идет о некоторых современных стратегиях антиадгезивной терапии, механизмах подавления адгезии патогена, иммунизации с использованием бактериальных адгезинов, адгезивных субъединиц или фрагментов иммуногенных пептидов и ДНК вакцин, кодирующих адгезин или его часть и т.д. Исследование бактериальных пилей, которые управляют колонизацией тканей хозяина, является областью, крайне нуждающейся в дальнейшем изучении. Бактериальные пили, будучи высоко иммуногенными структурами, находящимися под селективным давлением иммунного ответа хозяина, могут быть кандидатами в вакцины для многих важных патогенов человека. *C. diphtheria*, возбудитель дифтерийной инфекции, хорошо изучен в отношении токсинообразования, тогда как о его факторах, решающих для колонизации хозяина, известно мало. Адгезивные факторы *Corynebacteria* можно рассматривать как вероятные компоненты комбинированных дифтерийных вакцин с антибактериальным действием, которые локализуют дифтерийную инфекцию в организме. Они могут стать эффективным средством против стойкого бактерионосительства и будут способствовать прекращению циркуляции возбудителя в человеческой популяции.
Ключевые слова: адгезия, пили, *C. diphtheriae*, анти-адгезивная терапия, антибактериальный иммунитет, дифтерийные вакцины