

# New insights into bacterial persistence in reactive arthritis

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## ABSTRACT

*Persistence of arthritis-triggering bacteria can cause chronization of reactive arthritis (ReA). In the evaluation of bacterial persistence in ReA, the persistence of both the triggering bacteria and also of the other bacteria residing in the foci of chronic infection, are important. Two forms of bacterial persistence, cell wall-deficient bacteria (L-forms) and bacterial biofilms, are characterized, and the possible links between these forms and ReA are revealed. Data showing the possibility of bacterial ReA triggers to enter the cell wall-deficient state and to persist in bacterial biofilms, and evidence, suggesting that cell wall-deficient bacteria and bacterial biofilms are involved in the foci of chronic infection, are discussed. The understanding of the properties of microbes when they exist in cell wall-deficient state and bacterial biofilms may expand our knowledge on the clinical value of persisting microorganisms in ReA. In conclusion, both modes of persistence, cell wall-deficient state of bacteria and bacterial biofilms, deserve rheumatologists' attention, as their investigation, applying modern standardized methods, may contribute to the elaboration of new beneficial schemes of antibacterial ReA therapy.*

## Introduction

In rheumatology, the last decade has been marked by abundant attempts to elucidate the bacterial triggers of rheumatic diseases and ways of eliminating them using antibacterials. Most of these studies were assigned to reactive arthritis (ReA). ReA triggers of urogenital (1) and gastrointestinal (2) origin were found viable in the synovium of the host organism, however antibacterial therapy showed contradictory clinical results. A beneficial effect on the long-term prognosis of the disease was observed following a 3-month course of ciprofloxacin in the acute phase of

ReA (3). However, the results of other studies are different. Long-term ciprofloxacin (4), or lymecycline (5) treatment did not change the natural course of ReA. In addition, persistence of *Chlamydia trachomatis*, the most common trigger of ReA, was demonstrated in the synovium even after antibacterial treatment (6). Furthermore, the extended treatment with ciprofloxacin or ofloxacin not only failed to eradicate *Chlamydia trachomatis* from the host cells *in vitro*, but rather induced the state of chlamydial persistence, which was characterized by the presence of nonculturable but fully viable bacteria with altered steady-state levels of key chlamydial antigens (7). Obviously, the available data are still not sufficient to defeat the arthritis-triggering infection. This fact encourages attention to the unusual forms of bacterial existence, which assure their protection from the human immune system and a long-lasting persistence in the host. Persistence of triggering bacterial agents in ReA leads to the chronization of disease (8). The relapses of arthritis can be induced by other bacteria than the triggers of the disease (9), for example, those persisting in the focus of chronic infection. Elimination of such focus can essentially improve even an aggressive course of the disease (10). Thus, in the evaluation of bacterial persistence in arthritis, the persistence of both the triggers and of the other bacteria residing in the foci of chronic infection, seems important.

Clinical expression of the long-term presence of bacteria in the host depends on the genetics of the host and also on the particular environmental stresses on the bacteria and the host (11). When both host and bacteria, adapt sufficiently to overcome these stresses in order to survive, the relationships between them can become so successful that they both benefit and may evolve a sophisticated parasitism. The elucidation of such

time-serving microorganisms and evaluation of their pathogenicity and drug susceptibility require special methods that are different from those used for the parental pathogens. A challenge to the discussion among researchers and clinicians regarding the unusual biology of persisting pathogens and their relations to chronization of rheumatic diseases has been presented by Villarel and colleagues (12).

In the present review, we will discuss data on two forms of bacterial persistence – cell wall-deficient bacteria (CWDB), or L-forms, and bacterial biofilms, emphasizing the possibility that bacterial triggers of ReA and bacteria in foci of chronic infection may persist in a such manner. The terms CWDB and L-forms will be used interchangeably.

### Cell wall-deficient bacteria (L-forms)

#### *Characteristics of cell wall-deficient bacteria (L-forms)*

Many bacteria may develop as L-forms in which the cell wall is deficient in part or completely absent. Conversion of bacteria to a cell wall-deficient form was first reported for *Streptobacillus moniliformis* in 1935 by Klieneberger (13) and has later been documented for many other species of bacteria. Investigation of CWDB is still underway and seems to be gaining acceleration in rheumatology. All known bacterial species now seem capable of converting to CWDB under exposure to a variety of inducing agents (often to antibacterials). Removal of the inducing agent may result in reversion to the parental bacterial form or may not (14). It is no longer controversial that many bacteria can undergo spontaneous change to L-forms (11).

L-forms have lost their ability to construct peptidoglycan cell walls, which are replaced by the macromolecular components of the cytoplasm, bound as a cytoplasmic membrane and acting as a structural component (enzoskeleton). Despite that, they remain intact, grow, segregate their chromosomes and divide (15). Due to the lack of a rigid cell wall, L-form organisms generally possess various shapes; they may be small or large, spherical, irregular or

club-shaped (14). The key cell division protein, FtsZ, was detected in L-forms at a concentration one-fifth of that in the parental strain, and consequently may be too low for division to occur efficiently in the L-form (16).

CWDB are suggested to be intracellular organisms that can revert to wild-type bacteria outside the cell (11, 14). Mammals have developed a host defense system against microorganisms by sensing their surface materials. Thus, removal of the bacterial cell wall components may contribute to an evasion from the host defense system and the intracellular survival of those bacteria and ensure their long-term persistence. The term “persistence” has several aspects and its meaning is not the same for different species of bacteria. For instance, all *Chlamydia* species are obligate intracellular pathogens, and persistence for *Chlamydia trachomatis* and *Chlamydia pneumoniae* is perhaps a normal state required for a continued high-level transmission of these organisms (12). On the other hand, some extracellular pathogens, surface colonizers, actually seek an intracellular location also for persistency (17). For example, *Helicobacter pylori* can enter the host cells (18) and cycle between extra- and intracellular phases (19). Following the loss of cell walls, *Helicobacter pylori* L-forms lose certain antigens, possess more adhesiveness, invasiveness and tend to exist intracellularly. However, under certain conditions, they can revert to typical vegetative forms and cause a relapse of the disease (20). The rapidity of CWDB reversal to their parent form often correlates with the activity of disease (11). Investigation of gastric biopsies showed that both vegetative and L-forms of bacteria, can exist in parallel at the same *Helicobacter pylori* infection focus (21). A small number of L-form bacterial cells with a transient intracellular habitat is likely to serve as a seeder population, providing a backup for a constantly challenged and fluctuating luminal population (18).

For the detection of CWDB, special methods should be applied as they do not grow in the usual media. CWDB can be determined by a variety of tech-

niques such as electron microscopy, DNA detection, RNA detection, *in situ* hybridization, the presence of specific proteins and others (22–25). However, techniques used to identify CWDB are still not standardized. It is worth noting that DNA amplification could not be a tool for the differentiation of parent and cell wall-deficient forms of bacteria. Cell wall-deficient forms of *Mycobacterium tuberculosis* can be determined with the PCR kit for this pathogen, and the same band as for the parental forms is observed (26). The immune response produced by CWDB is different from that due to wild type bacteria (27). Thus, in addition to the serology of wild type bacteria, the presence of CWDB in disease and the dynamics of their survival after antibacterial treatment should be determined by specific tests for CWDB (28). For instance, in patients with chronic Lyme disease, following an extended antibiotic therapy, serologic diagnosis of *Borrelia burgdorferi* under the CDC/ASTPHLD recommendations was positive only in 4/47 patients (29). However, in the same study, by the use of special media for reversion of L-forms to parental forms, *Borrelia burgdorferi* have been cultured from blood in 43/47 patients, thus suggesting that using only serological tests, a vast majority of these patients would have been misdiagnosed as not having Lyme borreliosis.

Intracellular persistence is one of the mechanisms for L-forms to escape not only host defenses, but also action of antimicrobials, thus contributing to failures of treatment (18, 30). The loss of bacterial cell wall, so rarely demonstrated in clinical microbiology laboratories, is an important cause of antimicrobial resistance (31). CWDB are usually resistant to antibiotics under whose influence they have changed to L-forms (26); they are often resistant to other cell wall-active antibiotics and may also show alterations in sensitivity to other classes of antibiotics (31). The bioluminescence method can be used for a rapid detection of susceptibility to antibacterials in L-form bacteria and may play an important role in choosing the treatment (32). When the presence of CWDB is suspected in the pathogen-

esis of disease, clinical trials involving the use of combinations of bactericidal and bacteriostatic drugs, similarly to therapeutic interventions in tuberculosis, are suggested (28).

The presence of CWDB was demonstrated in infective endocarditis (22), rheumatic fever (33, 34), systemic lupus erythematosus (35), scleroderma (36), Crohn's disease (11), pyogenic arthritis (37), recurrent osteomyelitis (38), bone marrow transplants (39) and other diseases (14). CWDB isolated from clinical specimens do not always retain the identical characteristics of the parent bacterium, because a variety of biotypes that were presumably derived from a single genus and species in a given patient can be elucidated (11). The persistence of the L-forms of bacteria may lead to continuous low-level immunogenic stimulation and trigger immunopathologic events in the host (11, 33). Many of the so-called autoimmune diseases that represent immune reactions initiated by persisting CWDB might appear grossly overlooked and thus deserve further investigation (11).

#### *Cell wall-deficient bacteria (L-forms) and arthritis*

Experimental arthritis induced by a single intraperitoneal injection of *Eubacterium* cell walls into rats, closely resembles the findings of joint inflammation observed in human rheumatoid arthritis (40). In the latter *in vivo* model, out of the two tested bacterial strains that belong to the normal human intestinal flora and are 100% identical by 16S rDNA analysis, one proved to be arthritogenic and the other non-arthritogenic (41). The authors have concluded that the chemical structure of peptidoglycan in the cell wall is decisive for the arthritogenicity/non-arthritogenicity of bacteria. Thus, the loss of peptidoglycan cell wall, in the process of turning to the L-form and turning back to the parent form, seems to play an essential role in triggering arthritis and provoking its exacerbation.

A role of L-form ReA triggers in the chronization, relapses and response to the antibacterial treatment of arthritis is implied by some direct and indirect evidence.

Even several decades ago, Kagan and colleagues (42), in experiments on mice, showed that L-forms of group A streptococcus are implicated in the development of chronic streptococcal infection and postinfectious complications. Chronic tonsillitis (43) and chronic arthritis (44) were induced by group A streptococcal L-forms *in vivo*. CWDB were found in the focus of chronic infection and in the blood of experimental animals (33). In Wistar rats, intraperitoneally injected L-forms of *Streptococcus pyogenes* induced host response with atypically early increased numbers of monocytes and macrophages (45). However, despite the greater contribution of inflammatory macrophages to cellular response, they appeared to be ineffective in eliminating the cell wall-deficient streptococcal forms from the peritoneal cavity.

In humans, L-forms can also cause persistence of focal infection, maintain infection in the periods between recurrences and determine its new recurrences (46). L-forms of group A streptococci were isolated from the blood of 15.4% patients with chronic tonsillitis. In the blood of patients with rheumatic fever and with infectious endocarditis, CWDB were found with a significantly higher frequency (even in about 37% of patients) than the vegetative forms (33).

Several studies have indicated that most of the streptococcal carrier states are a kind of latent, atypical or inapparent infections and are associated with persistence of streptococcal L-forms inside the hosts (11, 14, 47). In contrast to the normal microflora, persistent pathogenic infection, even if it is asymptomatic, represents a burden for the carrier (17).

These studies are of particular importance for pediatric patients, where the most frequent site of residence of arthritis triggering infection is the upper respiratory tract (48, 49) and foci of chronic infection are also most often located in this region.

*Klebsiella pneumoniae*, widely known as a possible trigger of ankylosing spondylitis (50), by culturing was most frequently isolated during the active phase of disease (51). Clinical relapse

of disease was preceded by the appearance of *Klebsiella pneumoniae* in fecal samples (52). In our studies on several children with chronic arthritis and positive fecal samples for *Yersinia enterocolitica* (53) or *Escherichia coli* O1 (54), a 7-10-day antibacterial treatment caused recovery in all of those patients. Antibiotics earlier prescribed to these patients only on the basis of positive titers of *Yersinia enterocolitica* antibodies with negative fecal samples caused no changes in the clinical picture. An interpretation of these data other than the persistence of the mentioned Gram-negative gastrointestinal ReA triggers in L-forms is hardly possible. Thus, the beneficial effect of antibacterial therapy is likely to be expected exclusively if it is prescribed during the active stage of the disease when the triggering infection is culturable in the usual media. Every effort should be made to elucidate the bacterial trigger and to prescribe adequate antibacterials before the triggering infection has changed essentially and entered the persistent state. The beneficial effect of early antibacterial treatment has been already shown in children's ReA (49).

While discussing chlamydial persistence, Villarel with colleagues (12) have suggested, that it is promising to search for the possibilities to render situations with culturable infection not fortuitous and to find some ways to return the persistent organisms to the active developmental cycle, thereby making them more accessible to antibacterials.

Several efforts have been made to determine the sites of the persistence of triggering infection in rheumatic diseases, paying particular attention to bacterial triggers of ReA. In addition to the well established sites in joints, gastrointestinal or urinary tracts and lymph nodes (55, 56), unusual sites of the triggering infection persistence have been discovered. For instance, such pathogens as *Mycoplasma*, *Chlamydia*, some kinds of *Salmonella*, *Yersinia*, L-forms of group B streptococci can persist in the bone marrow for a long time; this probably allows them to escape the supervision of the immune system (57, 58). On the other hand, different groups of

antibiotics exhibit different abilities of penetration into the bone marrow. Thus, the bone marrow seems to be an underscored as a possible site of persistence of ReA triggers.

The presented data imply, that the persistence of infectious triggers of ReA in L-forms are waiting for today's reevaluation, especially as regards the expectations to elaborate schemes of more successful antibacterial treatment.

### Bacterial biofilms

#### *Bacterial biofilms – a mode of bacterial persistence*

The interest in bacterial biofilms has emerged in the last decade. The history of this form of bacterial persistence goes back to the 17th century when Anton van Leeuwenhoek scraped the plaque biofilm from his teeth and observed this microbial community with his primitive microscope. Recent advances have led to the current definition of bacterial biofilms as a structured community of bacterial cells enclosed in a self-produced extracellular polymeric matrix, adherent to an inert or living surface (59). In the environment, they are abundant in water-supply and sewer systems. Bacterial biofilms can be formed in human organisms and cause diseases. Biofilms also can complicate the course of nonbacterial diseases when they are formed on catheters, implants or other medical devices. They are important in the dissemination of various infections because during the formation of biofilm the pathogens form the "infective dose" (60).

Bacterial biofilms can be of different thickness and maturity and constituted from one or several species of microorganisms. For instance, in the biofilms of dental plaque, up to 500 bacterial species were found (61). Biofilms contain channels in which nutrients can circulate and their structure is adapted for long-term survival in a hostile environment. The higher the density of microorganisms in the biofilms, the more severe stress from fluctuations of temperature, pH, and limitation of nutrients they experience and the more alterations they undergo, including slowing down the growth rate, gene mutations and the development of new bacterial

phenotypes (62, 63). The intercellular bacterial communication mechanism that controls gene expression in response to population density is called quorum sensing. Quorum sensing was found to modulate the transformation of bacteria from planktonic to a biofilm mode of growth (64). In addition to induction of biofilm formation, quorum sensing signals converge with starvation-sensing pathways to regulate cell entry into the stationary phase (65). In different regions of biofilm, cells have different phenotypic composition and exhibit different patterns of gene expression (66). By phenotypic composition every bacterial biofilm is unique. The structure, metabolism and functions of bacterial biofilms are so complicated that they resemble the tissue of higher organisms (67).

The influence of the factors of innate immunity on the biofilm formation has recently been investigated, focusing attention on lactoferrin. Lactoferrin is usually found in tears, respiratory tract secretion and in high levels in mother's milk (68). In addition to bactericidal and bacteriostatic effects, lactoferrin exhibits a suppressive effect on bacterial biofilm formation (69). The formation of biofilms is inhibited by low concentrations of lactoferrin, much lower than the bacteriostatic and bactericidal levels. However, lactoferrin has no effect on mature biofilms. It binds iron, thus allowing bacteria to flow easily on the mucous surface without rallying into biofilms (69). Free iron is required for the growth of bacteria, whereas much larger amounts of iron are needed for biofilm formation.

#### *Susceptibility of bacterial biofilms to antibiotics*

Bacteria in biofilms are 10 -1000 times more resistant to antimicrobial agents than in planktonic form, and this feature is determined by many factors (70). A high density of bacteria and the exopolysaccharide matrix prevents the penetration of antimicrobials, and the response to antibiotics depends on the depth at which bacteria are found in a biofilm (71). Moreover, the penetration also depends on the type of antibiotics as well as on the type of bacteria com-

posing a biofilm. For instance, ampicillin can penetrate through a biofilm formed by the  $\beta$ -lactamase-negative strain of *Klebsiella pneumoniae* but not through a biofilm formed by  $\beta$ -lactamase-positive wild strain of the same microorganism (72). When bacteria slacken their growth rate as happens in biofilms, their resistance to antibiotics increases (73). Bacteria can exchange plasmids by conjugation within biofilms at significantly higher rates than under planktonic conditions, and the factors of antibacterial resistance might be carried with those plasmids (74). When the biofilm-specific phenotypes of bacteria are stimulated, the biofilm-specific resistance mechanisms are expressed (75, 76), and they are different from the mechanisms of planktonic bacteria (77). As bacteria in a biofilm are heterogenic, in the same biofilm several mechanisms of resistance to antibacterials can take place (78).

The above data encouraged the search of new approaches to antibacterial therapy of biofilms, including their application in the form of liposomes (79), cyclical application regimen (80), the biofilm matrix- or intercellular signaling-targeted treatment (81). The fact that bacteria can develop reduced susceptibility to antibacterials even in the very thin biofilms can be explained by the presence of a subpopulation of persisters (82). This small number of bacterial cells, which have remained after the application of antibiotics, can be eliminated with the second antibacterial course prescribed at a later period after the first course (80).

An alternative approach to biofilm control is to target the matrix of a biofilm, either by inducing the destruction of the matrix polymers or by blocking their synthesis (81). For instance, macrolide antibiotics exhibit therapeutic efficacy against some lung infections, even though these agents are only weakly bactericidal. These antibiotics damage biofilms formed by those microorganisms by reducing matrix polysaccharide synthesis (83). Quorum sensing systems have emerged as an enticing target for fighting biofilm infections due to their role in coordinating biofilm formation and activating virulence factors in many



Gram-negative and Gram-positive bacteria (84). Therefore, analogues of the natural signal molecules that jam bacterial communication could prove to be effective antibacterial drugs (81), and this strategy has shown some promise in laboratory tests (85). Antimicrobials based on quorum sensing interference would have a high specificity against the target organisms, leaving beneficial commensal bacteria unharmed during therapy, with a minimal likelihood of antibiotic resistance being transmitted between species. With the inevitable decline of the effectiveness of currently used antimicrobials these novel approaches should be seriously considered (84). As bacteria of young biofilms are more susceptible to antimicrobials than old ones, preparation of the new non-invasive methods for detection of young biofilm formation could be useful, and several laboratories are now making attempts to elucidate the genes that are activated or suppressed at the very beginning of biofilm formation (86, 87).

#### *Bacterial biofilms as a cause of human diseases*

In a human organism, bacterial biofilms can grow on the live tissues (as in infective endocarditis), dead tissues (as on bone sequestra) and on medical devices. Biofilms grow slowly in one or several places and induce persistent clinical symptoms (88). The sessile bacteria of the biofilm release antigens and stimulate the production of antibodies. These antibodies are not able to kill bacteria within the biofilms, however, they may cause the immune complex-triggered damage of surrounding tissues (89). Bacteria within biofilms which dwell on medical devices produce endotoxins, which may elicit an immune response in the patient (90). Before the induction of clinical symptoms, biofilms have been growing for months and even for years. Due to the reduced growth rates of bacteria in biofilms, the diagnosis of biofilm-triggered diseases by culturing is often complicated and requires the application of more sensitive methods, such as polymerase chain reaction (PCR) or others (91, 92). Antimicrobial therapy usually kills the

freely floating bacteria in the organism and thus eliminates symptoms of a biofilm-induced disease, whereas it is not able to destroy the biofilm and to cure the disease completely (86, 93). For the latter reason, symptoms of biofilm-triggered infections usually relapse after discontinuation of even a long-lasting treatment with antibiotics.

In some diseases the pathogenetic link with bacterial biofilms has already been established, while in others it is still under investigation. This link is most thoroughly studied in infective endocarditis (93) and in the damage of the respiratory system in cystic fibrosis (94). Formation of bacterial biofilms has been demonstrated also in clinical isolates of adult chronic obstructive pulmonary disease (95), osteomyelitis (96), biliary tract infection (97), cholelithiasis (98), urinary stone genesis (99), bacterial prostatitis (100), periodontitis (61), ophthalmic infections (101), various kinds of catheters, implants and other orthopedic devices (90, 92). By PCR, prosthetic hip infection was determined 3.5 times more often than by the culture method, and this difference could be due to bacterial growth in biofilms, suggesting that in many cases the so-called aseptic prosthesis loosening might actually be caused by the undetected biofilm infections (92). Moreover, this form of bacterial persistence is also likely to be implicated in rheumatic arthritides. However, to the best of our knowledge, this link has never been investigated.

#### *The possible link of bacterial biofilms with reactive arthritis (ReA)*

In ReA, the importance of biofilms could be discerned at least in two aspects. The first aspect is the presence of bacterial biofilms in foci of chronic infection, the other aspect being the possibility that potential bacterial triggers of arthritis can form biofilms (Table I) and thus assure their long-lasting persistence in the organism.

The link between gastrointestinal ReA triggers and the biofilm mode of bacterial persistence could be demonstrated on *Salmonella* infection. Three to five per cent of the people infected with *Salmonella enterica* serovar Typhi

become chronic carriers of the micro-organism, and this state is frequently associated with gallbladder abnormalities, such as gallstones, most of which do not cause any symptoms (97). The vast majority, if not all, gallbladder carriage of *Salmonella* spp. involves biofilm formation on fully formed gallstones, newly nucleated pregallstones, or other hepatobiliary abnormalities (98, 102). Antibiotic treatment is often ineffective in *Salmonella* carriers with gallstones, and elimination of gallbladder infection in these individuals usually requires surgery and gallstone removal in adults (97) and in children (103).

Furthermore, ceftriaxone itself is known to induce reversible precipitates in the gallbladder of adults and children. These precipitations have a striking similarity to gallstones on ultrasound examination and are called pseudolithiasis. Pseudolithiasis develops in 10–40% of patients treated with ceftriaxone (104). It may occur from the first days of treatment, most often is asymptomatic and resolves in 2–63 days from the end of treatment (105, 106). The fate of such precipitations in patients with arthritis (for example, with Lyme arthritis, for which ceftriaxone treatment is widely used) is difficult to predict. Considering that there are no targeted studies on the possibility of biofilm formation, resulting in a focus of persistent infection on these precipitates, it seems reasonable to follow these arthritis patients till the disappearance of pseudolithiasis.

Data on the relation of other possible bacterial ReA triggers and biofilms are also not abundant. Biofilm formation was demonstrated by *Yersinia pseudotuberculosis* on a biotic surface (107), and by *Mycoplasma* species in the environment (108).

*Klebsiella pneumoniae*, a potential triggering agent of spondyloarthritis (50), is also prone to form biofilms (109) which are resistant to killing during prolonged exposure to ampicillin or ciprofloxacin (110). There is a probable link between the capability of *Klebsiella pneumoniae* to grow in biofilms and the insufficient efficacy of these antibacterials in clinical stud-

**Table I.** Implication of ReA triggers in the formation of bacterial biofilms.

Bacteria	Site of bacterial biofilm formation	Comments	References
<i>Salmonella</i>	gallstones	in adults <i>in vitro</i>	102 98
<i>Klebsiella pneumoniae</i>		<i>in vitro</i>	109, 110
<i>Yersinia pseudotuberculosis</i>	biotic surface	<i>in vivo</i>	107
<i>Streptococcus pyogenes</i>	tonsils	in children's chronic tonsillitis <i>in vitro</i> (cultures from children with pharyngeal infection)	118 117
<i>Haemophilus influenzae</i>	middle ear	in children's middle ear infections in experimental animals	122 121
<i>Chlamydia trachomatis</i>	indirect evidence from the treatment results in adults: in undifferentiated spondyloarthritis in chronic prostatitis		131 111

ies of arthritis. *Chlamydia trachomatis* seems to take part in biofilm formation, which might lead to the failure of common antibacterial therapy in chronic prostatitis (111).

In children, the onset of ReA in more than half of patients is associated with the clinical presentation of upper respiratory tract infection (48, 49). *Streptococcus pyogenes*, the predominant bacterial pathogen in pharyngitis and tonsillitis, is the most frequent upper respiratory tract-derived trigger of ReA in children and adults (112-114). Almost all strains of *Streptococcus pyogenes* are susceptible to penicillin *in vitro*, but despite that, treatment failure rates in pharyngitis reaches up to one-third in clinical practice (115-116). Conley *et al.* (117) examined A group streptococcal cultures from 99 children (aged 2-18 years) with symptoms of pharyngeal infection and demonstrated that all these microorganisms are able to form biofilms *in vitro*. However, the authors conclude that the capability to form a biofilm alone does not explain insensitivity to penicillin and subsequent penicillin treatment failure. The synergistic effect of indirect pathogens, such as *Maraxella catarrhalis* or non-typeable *Haemophilus influenzae*, could be expected in streptococcal biofilms which become multispecies as they develop, incorporating other species of bacteria in the biofilm in order to create a mutually beneficial relationship for survival (117). Investigation of the ultrastructure

of the tonsils, surgically removed due to recidivating tonsillitis has demonstrated the anatomical evidence of microbial biofilms in tonsillar tissue, which might serve as a possible explanation for the chronicity and recurrent nature of some forms of tonsillitis (118). Of note, biofilm formation by clinically relevant serotypes of group A streptococci depends on their serotype, and isolates belonging to the same serotype were found to be very heterogeneous in the biofilm-forming behaviour (119).

Chronic tonsillitis is the most common and representative focus of chronic infection in children, but also the other foci of chronic infection in the upper respiratory tract may have an influence on the course of arthritis. The nontypeable *Haemophilus influenzae* can cause children's otitis media and sinusitis and is implicated in the development of ReA (120). Bacterial cultures in otitis media often are negative but reverse transcription PCR-based assays have shown the presence of bacterial mRNA, indicating that bacteria are present in a viable and metabolically active but nonculturable state (91, 95). Experimental and clinical data confirmed *Haemophilus influenzae* biofilm formation in recurrent otitis media (121, 122).

When acting as direct or indirect pathogens and forming biofilms, bacteria change their properties and especially their susceptibility to antibiotics (123). Investigation of biofilms is widely used in the elaboration of antibacterial

prophylaxis and treatment methods in infections related to prosthetic joints and other medical devices. Rifampin and its new derivatives recently attracted particular attention and are common constituents of antibiotic combinations against biofilm infections (124-126). Rifamycins are extremely effective in the treatment of latent *Mycobacterium tuberculosis* infection (127), but their effects are not specific to mycobacteria. Rifampin and related drugs are active against a variety of Gram-positive and Gram-negative organisms, including *Streptococci*, *Enterococci*, *Staphylococci*, *Neisseria spp.*, and members of family *Enterobacteriaceae*; their potencies against both sexually transmitted *Chlamydia trachomatis* and respiratory tract pathogen *Chlamydia pneumoniae* are of particular interest for rheumatologists (128). Rifampin demonstrated excellent bactericidal activity against young or mature biofilms probably due to its potential to reduce adherence of the biofilm organisms to the surfaces (128), and its activity against bacteria in all phases of growth, including actively growing, semidormant, nongrowing, and intracellular (128, 129). Novel rifamycin derivatives with prolongation of action and compounds acting on bacterial quorum sensing mechanisms now are under investigation (124).

Rifampin is effective when combined with ciprofloxacin and tetracyclines, the antibiotics which are most often used in the studies of antibacterial treatment efficacy in ReA. In combination with ciprofloxacin, rifampin was shown to be effective in the animal model of otitis media caused by nontypeable *Haemophilus influenzae* biofilms (130). Carter *et al.* (131) demonstrated therapeutic benefit with antimicrobial complex consisting of doxycycline and rifampin for 9 months even in patients with chronic undifferentiated spondyloarthritis (average disease duration 10 years) with special reference to *Chlamydia*-induced arthritis. It seems, that such new antibacterial complexes could also be beneficial in the treatment of rheumatoid arthritis, which is thought to be a heterogeneous disease and has an etiologically

overlapping area with ReA (132).

The presented data suggest that arthritis triggering and the other bacteria in the foci of chronic and recurrent infection are linked to biofilm formation, and controlling arthritis with antibacterial agents requires the application of knowledge on bacterial biofilms.

### Comparison of CWDB (L-forms) and bacterial biofilms

CWDB have lost their cell wall and tend to exist intracellularly. Biofilms are matrix-encased bacterial communities that are specialized for surface persistence, and the hallmark of bacterial biofilms that segregates them from bacteria that are simply attached to a substratum is that biofilms contain extracellular polymeric substances that surround the resident bacteria (133).

The reviewed data reveal several similarities between CWDB and bacterial biofilms. Both of those forms of bacterial persistence developed specific ways to avoid eradication by the immune system, mostly by disrupting contact of bacterial antigens with the immune system. In a similar way, these forms can survive after treatment with antibacterials. In both cases there remains a small population of survivors that, under certain conditions, can restore the infection. The elucidation of causative bacteria in both of those forms is often complicated as routine bacterial culture methods or serology tests are not sensitive enough to detect them, indicating that the associations of diseases with causative bacteria in many cases remains undiscovered. This results in the lack of appropriate treatment and diseases take a chronic course.

Comparison of CWDB and bacterial biofilms divulge that both these forms of bacterial persistence can be located in the same sites in the organism forming foci of chronic infection (33, 118) or other disorders. For instance, CWDB are found within cardiac valvular vegetations, which are typical examples of bacterial biofilms in infectious endocarditis (22, 93). Finally, two studies investigated both of these forms simultaneously and showed by bioluminescence method *in vitro* that

L-forms of *Listeria monocytogenes* can form biofilms, as parental bacteria do (134, 135).

### Conclusion

In the evaluation of bacterial persistence in ReA, the persistence of triggering bacteria and also of other bacteria residing in the foci of chronic infection seems important. It is highly probable that both presented forms of bacterial persistence, CWDB and bacterial biofilms, are implicated in the modulation of the course of ReA. However, targeted studies are needed to address these issues. In such investigations, the application of modern standardized methods is required. The understanding of the properties of the microorganisms persisting in a cell wall-deficient state and in bacterial biofilms expand our knowledge on the clinical value of these microorganisms and may contribute to the elaboration of new beneficial schemes of antibacterial ReA therapy.

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