Is Bacteriolysis *In vivo* a Friend or a Foe? Relation to Sepsis, Chronic Granulomatous Inflammation and to Oral Disorders: an Overview Hypothesis

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Abstract

The possible role of antibiotics and of leukocyte-derived cationic peptides as inducers of bacteriolysis in Gram positives and Gram negatives and their involvement in the pathogenesis of chronic granulomatous inflammation is briefly reviewed. It can be speculated that in humans, leukocytes laden with intracellular bacteria and their non-degraded highly-phlogistic cell-walls may be translocated from inflamed gums (periodontal disease) and from infected dental pulps (pulpitis, periapical granulomas) to remote sites such as damaged heart valves (causing endocarditis) and injured joints (causing chronic arthritis). This phenomenon, maybe important, clinically and is in line with the old "Focus of infection theory" from the nineteen twenties, which is no longer considered and discussed in the modern literature.

**Keywords:** Bacteriolysis; Antibiotics; Cationic peptides; Translocation; Oral infections; Perpetuating chronic inflammatory processes

Prologue

Today there is still an ongoing failure to offer an effective treatment for serious sequelae which too often develop following the invasion of the blood stream by microorganism causing severe sepsis and septic shock. Although Gram-positive and negative pathogens are the most common causes of sepsis, fungal involvements in sepsis are rapidly on the rise. It is alarming that according to CDC, the annual incidence of sepsis in the USA affects as many as 750,000 hospitalized patients and the mortality rate is about 40% [1,2]. Although septic shock is strongly associated with microbial infections, bacteria in the blood are found only in about 50% of cases of severe sepsis and septic shock, and 20% to 30% of patients will have no microbial cause identified from any source. However, the possibility should be considered that pathogenic microorganisms may hide in the liver, spleen or in the lung and therefore will not be isolated in blood cultures.

The initial reactions to infection are generalized pro- and anti-inflammatory responses. This usually begins by activation by microorganisms and some of their products of neutrophils, macrophages and monocytes; which is followed by effects on vascular endothelial cells via pathogen recognition receptors. This may lead to host responses and to the mobilization of the complement system, resulting in endothelial cells disruption. It was recently suggested that histones, released from leukocyte nuclei, may be major mediators of death in sepsis [3,4].

Since sepsis may be caused mainly by Gram positives and by Gram negatives, the question was addressed whether we deal with two different clinical manifestations [5]. The authors stated that:

"It has been assumed that the initiation of the systemic inflammatory response with activation of the pro inflammatory cytokine networks and other mediators results in a similar organisms. Systemic immune activation during sepsis may promote the clearance of the microbial pathogen; however, generalized inflammation also contributes to the pathogenesis of septic shock. The balance between these beneficial and deleterious effects may differ between Gram-positive and Gram-negative pathogens pathophysiologic process, regardless of the causative microbicpathogen. Yet, there is increasing experimental evidence that fundamental differences exist in the host response to Gram-positive bacterial pathogens, compared with the host response to Gram-negative."  

The main pro inflammatory agonists that may be released into the blood stream following lysis of microorganisms include:

- Cell surface Lipopolysaccharides (LPS) from Gram negatives
- Membrane-associated Lipoteichoic Acid (LTA) from Gram-positives
- Capsular polysaccharides from pneumococci
- Microbial hemolysins and additional membrane-damaging agents
- The host may supply: toxic amounts $\text{H}_2\text{O}_2$, hydroxyl radical, HOCl, Nitric Oxide (NO) and peroxynitrite, Lysosomal acid hydrolases, bactericidal cationic peptides
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Is Bacteriolysis *In vivo* a Friend or a Foe? Relation to Sepsis, Chronic Granulomatous manifestations. It is therefore alarming that despite the recognition that post-infectious sequelae may result from a "cross-talk" among a multiplicity of many of these agonists [1,2], to date, all the clinical trials in humans that had tried to alter the course of sepsis by testing only a single antagonist at a time, had failed. It is therefore of great concern that today, even activated protein C, the great hope for sepsis treatment, yielded poor result and the agent was recently removed from clinical use [6]. A strong support for the assumption that the pathophysiology of severe infections and post-infectious sequelae may involve a "cross-talk" among a multiplicity of pro-inflammatory agents, but not due to a single agonist, emerged from extensive studies conducted since the early 1970s on the pathophysiology of diseases caused by group A hemolytic streptococci [7] and later on the role of cationic peptides and of certain antibiotics as activators of bacteriolysis [8-15], which may all aggravate the outcome of post-infectious sequelae. It is therefore highly likely that multi-drug strategies, yet to be developed, might better cope with insults caused by cross-talks among a multiplicity of pro-inflammatory agents [16].

The present overview stresses the assumption that non-biodegradable microbial cell-wall components released following bacteriolysis may play a pivotal role in cell and tissue damage observed in chronic inflammatory and granulomatous manifestations.

**What is Bacteriolysis?**

**Historical notes**

The discovery of microbial cell killing by serum factors dates back to 1883, when Buchner had reported that fresh serum was able to kill certain bacteria but that this property was lost upon heating serum to 56°C. One year later, Pfeiffer reported the bactericidal dissolution ("Pfeiffer's phenomenon": bacteriolysis) of cholera vibrio by fresh serum which could be correlated with protection against infection. In 1895 Bordet showed that two factors, a heat-stable (antibodies) and a heat-labile (Alexin) were required for this reaction. Alexin is today known as complement (reviewed By gladsone, et al. 1964) [17]. However, two important events in the field of microbial cell killing and degradation were the discovery in 1928 by Alexander Fleming of the enzyme lysozyme [18] and later on of penicillin [19].

**Bacteriolysis: How is it Triggered and How it May Be Involved in Cell and Tissue Damage?**

Bacteriolysis is an event in which the rigid cell-wall of bacteria, comprised of a polymer of N-Acetyl glucosamine – N-Acetylmuramic acid (Peptidoglycan-PPG), is split, allowing the release of intracellular cytoplasmic components, due to the high osmotic pressure of the cell sap [10-12]. Morphologically, two main patterns of microbial cell-destruction can be identified:

- The term ‘plasmolysis’ is proposed when, either by electron microscopy or by radio-labeling microbial components, a significant degradation of cytoplasmic constituents had occurred but without significantly affecting the structure of the PPG.
- The term ‘bacteriolysis’ was proposed to indicate a significant breakdown and degradation of the PPG, presumably due to the uncontrolled activation of intracellular autolytic wall enzymes [11,12,15].

It should, however, be stressed that killing of bacteria either by the immune system (antibodies and complement) or by phagocytes, precedes the phenomenon of bacteriolysis. Therefore, even non-viable bacteria may still contribute to inflammatory responses by supplying pro-inflammatory non-biodegradable cell-wall components. Anecdotally we wonder: "can some life be still left after microbial demise?"

The main biological significance of bacteriolysis in vivo is the presentation of microbial antigens to the immune system. However, the uncontrolled release into the blood stream of microbial capsular polysaccharides, LPS, LTA, PPG, intracellular cytolsins, and histone from neutrophils is probably the main reason for patients' demise. It is conceivable therefore that agent/s, capable of attenuating bacteriolysis in vivo, might be useful to control some of the sequelae of microbial infections especially during antibiotic treatment and in chronic granulomatous episodes (see below).

Lysozyme, the “magic enzyme”, secreted by phagocytes, was thought to function to directly cleave bacterial peptidoglycans (PPG), leading to bacteriolysis. However, this was found true mainly for non-pathogenic *micrococci* used today as a substrate for lysozyme, but is not relevant to other microbial species. It turned out that unlike *Micrococci*, which possess a "naked" PPG, *Staphylococcus aureus* and additional pathogenic microorganisms could not be directly lysed by lysozyme because of the presence of O-acetyl groups bound to the peptidoglycan [20,21]. Furthermore, it was demonstrated that the lytic activity of lysozyme was not associated with its enzymatic activity but due to its high cationic charge [15,20-23]. It was therefore not surprising that spermine, spermidine, chlorhexidine, polymyxin B, the enzymes myeloperoxidase, ribonuclease, phospholipase Aₙ and elastase, which are all highly cationic, had the ability to lyse *Staphylococci* by activating their intracellular autolytic wall-enzymes, a “Trojan-Horse” phenomenon. Using combinations among lysozyme, H₂O₂ and ascorbic acid at pH 7.0 showed that the oxidation of ascorbic acid by hydrogen peroxide gave rise to a very potent antibacterial mechanism, which in presence of lysozyme, induced lysis of Gram negatives [24].

However, when comparing bactericidal and bacteriolytic phenomena, it is important to stress that unlike the short time (minutes) needed to kill bacteria by cationic agents (see review [15], the breakdown of the PPG by cationic peptides - induced autolysins, may take several hours and is optimal at pH 5.0, a condition which increases protonation of polycations. While the regulation of the autolytic wall-enzymes in Gram positives...
Bacteriolysis Induced by Antibiotics

The extensive use of beta-lactam antibiotics in clinical settings raises the question whether antimicrobials possessing bactericidal or bacteriostatic effects might also act as bacteriolysis-inducing agents (Figure 1). This might be important clinically since microbial lysis may induce the Jarisch-Herxheimer syndrome and there are also evidences from animal models and clinical studies of sepsis that antibiotic-mediated release of biologically-active cell-wall components from Gram-positive, Gram-negative or from fungi is associated with a rapid clinical deterioration. Therefore, the antibiotics selected to treat septic patients should be carefully considered [27-29]. One study [30] tested nine antibiotics, representing seven classes, for the amounts of endotoxin released during their action on susceptible strains of Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, and Pseudomonas aeruginosa. Aztreonam induced the highest release of endotoxin, whereas other antibiotics, such as imipenem and the quinolones, induced the lowest release of endotoxin. It was also stated that:

*although the quantities of endotoxin released are not easily explained from the established mechanisms of antibiotic action, our findings may have implications for therapy of the acutely ill septic patient in whom release of large quantities of pro-inflammatory agents may occur.*

What might be the Disadvantages if Bacteriolysis In Vivo is Blocked?

Since cationic agents such as lysozyme, LL37, histone and certain antibiotics can act as bacteriolysis-inducing agents, what might be the side effects if bacteriolysis in vivo is interfered with? It was shown that in vitro, the sulfated compounds heparin, polyanethole sulfonate and Evan’s blue markedly depressed lysis of Staphylococci induced by penicillin [31], lysozyme [32] and by complement [33]. Polyanethole sulfonate is routinely added to blood cultures to prevent killing and lysis of Gram-negative by complement thus securing their successful isolation from blood [34]. Lysozyme lysis of Streptococcus sanguis was also shown to be blocked by chitin oligosaccharide [23]. Therefore, these findings suggest that sulfated polysaccharides in infected connective tissues might perhaps modulate bacteriolysis induced by complement, cationic peptides and also by certain antibiotics to allow the persistence of non-biodegradable microbial cell-walls, causing chronic inflammatory episodes [15].

It is accepted that microorganisms may be killed and removed after phagocytosis by PMNs and by macrophages functioning mainly on mucosal surfaces and in tissues. However, it was also shown that during systemic inflammatory responses in severe sepsis, neutrophils accumulated in the liver microcirculation and...
Neutrophil Extracellular Nets (NETs) released into the vasculature ensnared bacteria from the bloodstream and prevented their dissemination, this required platelet-neutrophil interactions [35,36]. However, it was also suggested that phagocytized bacterial cell-walls might be translocated to remote tissue sites where they can persist for long periods to induce secondary foci of chronic granulomatous inflammation [37,38]. It is also possible that in chronic inflammatory conditions macrophages laden with bacterial remnants (PPG) may be retained in situ due to Macrophage Inhibiting Factor (MIF) [39] and to induce granulomas such as seen in mycobacterial and fungal infections (see below). Experimental granuloma formation can also be induced by injecting cell-walls and PPG from different microbial species [15] (Figure 1).

One additional explanation why phagocytes may fail to degrade microbial PPG is the possibility that H$_2$O$_2$ and proteinases delivered into leukocyte phagosomes following phagocytosis, might destroy microbial autolytic enzymes [40]. This effect could totally be reversed by catalase and by protease inhibitors, respectively. It was also shown that myeloperoxidase released by activated neutrophils, prevented the lytic effect of penicillin [41]. Clindamycin retarded the lysis of Staphylococci in macrophages due to the formation of extra thick walls rich in O-acetyl groups bound to the PPG [42].

**Bacteriolysis and Arthritis**

The inability of phagocytes to effectively biodegrade bacterial and fungal cell-wall structures may lead to granulomatous chronic inflammation in infections caused by *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Pseudomonas species*, *Nocardia species*, and fungi such as *Candida albicans* and *Aspergillus species*. The use of experimental models, mostly in rats, showed a long persistence of microbial cell-walls residing within large macrophages due probably to their inability to degrade the PPG [43-48]. Such stimulated macrophages probably release into the surrounding media chemotactic agents, enzymes and cytokines, which can amplify and also further perpetuate the inflammatory responses (Figures 1 and figure 2).

**Are Microbial Cell-Walls also involved in the Pathophysiology of Periodontal Disease and in Pulpitis?**

Periodontal disease is an inflammatory affliction of the gums which affects large numbers of the community. It is characterized by a protracted and progressing inflammation of the gingival tissue which, if not treated, might lead to regression of gums, bone loss and eventually to loss of teeth. The main pathogens which are involved in the pathogenesis of periodontal disease are the catalase-negative anaerobes *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Actinobacillus actinomycetemcomitans*, and a cross-talk among bacteria, the immune and inflammatory processes are probably the main causes of periodontal disease (Ginsburg, et al. [48], Lamont & Jenkinson, [49]).

**The Pivotal Role of Endotoxin (LPS) in Tissue Damage and in Septic Shock**

As stated by Lamont, et al. in 1998 [49] and by Medianos, et al. in 2005 [50].

"the bacterial components/virulence factors involved in periodontal disease may involve modulating inflammatory responses, which include responses mainly to Lipopolysaccharides (LPS) shed from microbial surfaces by complement, Peptidoglycans

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**Figure 2:** TEM image of a large macrophage in a mouse thigh muscle lesion ten days following injection of *Staphylococcus aureus*. Note the presence of large number of cell-walls in the cytoplasm, which indicates the inability to degrade peptidoglycan. (b) A cluster of macrophages laden with cell walls obtained from the same specimen. Such macrophages can release a large variety of inflammatory mediators and oxidants. Note that the gray areas within the cell walls are due to the fixative (Magnifications: x10260 for (a) and x5586 for (b)).

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(PPG), proteases, heat-shock proteins, the highly chemotactic formyl-methionyl peptides, and toxins. Inflammatory responses also involve: Toll-like receptors (tlr5), CD14, nucleotide-binding oligomerization domain proteins (Nod) and G-protein-coupled receptors, including formyl-methionyl peptide receptors and protease-activated receptors. Of the above bacterial and host molecules, evidence from experimental animal studies implicate LPS, fimbriae, proteases, TLRs, and CD14 in periodontal tissue or alveolar bone destruction”.

However, as further stated

“evidence verifying the involvement of any of the above pro-inflammatory agents in periodontal tissue destruction in humans, does not exist”.

Despite such skepticism, complement-mediated LPS (endotoxin) released from the anaerobes most probably plays a pivotal role in recruiting neutrophils to sites of inflammation where they can induce cell injury in the periodontium and tooth pulp [51]. Experimentally, Lipopolysaccharides (LPS) from Bacteroidesoralis and Veillonella parvula and cell-wall material from Lactobacillus casei were studied for their capacity to induce leukocyte migration into the dental pulp and also in an implanted wound chamber [52]. High molecular weight complexes of bacterial cell-walls may adversely affect pulpal tissue across freshly-exposed dentin. The morphology of human dental plaque has been studied by electron microscopy. In the inner part of the plaque, especially in the region adjacent to the tooth surface, many microorganisms exhibited unusually thick cell-walls, which contained large amounts of intracellular polysaccharide. The magnitude of both phenomena diminished towards the saliva-plaque interface. Sepsis with multiple-organ failure has traditionally focused on lipopolysaccharide of Gram-negative bacteria, the classical endotoxin. However, Gram-positive sepsis now accounts for up to 50% of all cases, calling for a shift of focus. Peptidoglycan (PPG) which is the major cell-wall component of Gram-positive bacteria can reach the circulation following bacterial breakdown or translocation from the intestine. Administration to animals of PPG induced all the classical features of infectious illness and endotoxemia and may cause systemic inflammation with organ failure [53]. Lipoteichoic Acid (LTA), the regulator of autolysis shed from Gram-positives by antibiotics by lysozyme or also following induction of bacteriolysis by cationic peptides, might also contribute to cell damage [12,54]. Neutrophils treated by LTA-anti LTA antibodies, generated large amounts of superoxide and H₂O₂ [55]. Lipoteichoic Acid (LTA) released from cariogenic streptococci dwelling adjacent to gingival pockets, might also induce bone resorption [56] and when streptococci were treated with glucose, they also generated large amounts of thiocyanic acid [57]. However, unlike in Gram positives, electron microscopy of the Gram negative E. Coli, treated by complement sufficient serum, revealed only outer-membrane disruption in the form of blebs but the inner microbial membrane and the rigid cell-walls remained intact [58]. Of interest is the ability of cationic lysozyme could detoxify LPS [59].

Taken together, it suggest that similarly to Gram positives, un-degraded PPGs from Gram-negatives might persist for long periods to contribute to the propagation of the inflammatory and destructive processes seen in periodontal disease. The activation of neutrophils in inflamed site might also generate HOCl capable of injuring tissues. Since similarly to sepsis, periodontal disease is most probably caused by a synergistic cross-talk among a multiplicity of pro-inflammatory agents, it stands to reason that multidrug strategies [16], yet to be devised, might be more efficient to suppress tissue damage than the use of single antagonists. Since survival of microbial cell-wall component in tissues may contribute to chronic inflammatory episodes, it is surprising that only a few studies have attempted to identify the possible presence of non-biodegradable microbial cell walls in the inflammatory bed either in the infected periodontium or in dental pulp (Figure 1). Already in 1979, John Schwab, et al. (reviewed in [11,45,46] had proposed for search for bacterial cell-wall components in chronic infected and inflammatory sites. Since microbial Peptidoglycan (PPG) is composed of a polymer of N-acetylglucosamine - N-Acetylmuramic Acid (NaG-NAM) and since muramic acid is a unique microbial agent, not found in mammals, its identification in the inflammatory bed may indicate the presence of bacterial cell-walls, which can act as potent activators of the immune and inflammatory responses. Also, detecting muramic acid in the dental pulp and in periapical granulomas might be indicative of presence microbial remnants.

Can Catalase-Positive Commensals and Red Blood Cells Secure the Survival of Perio-Pathogens?

A possible role of the catalase-positive commensals Candida albicans, Staphylococcus aureus, E. Coli and Pseudomonas aeruginosa, and also of red blood cells as protectors against H₂O₂ of the catalase-negative perio-pathogens Porphyromonas gingivalis and Fusobacterium nucleatum should be considered. This may be important especially since Chlorhexidine (CHX) is widely used in the oral cavity as a bactericidal agent. In the oral cavity, bactericidal amounts H₂O₂ may be produced by cariogenic streptococci grown with glucose [57], and also by mitochondrial respiration and may also be supplied by certain tooth pastes. Therefore, paradoxically perhaps, commensals and catalase-rich red blood cells may both protect strict catalase-negative anaerobes, against H₂O₂ toxicity (to be published).

Regarding CHX action, recent observations (to be published) suggested that Micrococcus lysodeikticus, the substrate for lysozyme, when coated by CHX, becomes highly-resistant to lysozyme action. Since highly catalytic CHX avidly binds to surfaces of many microorganisms, it is possible that treatment with CHX might paradoxically prolong the survival of microbial cell-wall components in tissues.

Since CHX was also found to increase the permeability for H₂O₂ of many commensals in the oral cavity and to enhance the decomposition of H₂O₂, it might also protect catalase-negative anaerobes against peroxide, leading to their prolonged survival in deep pockets of the gingiva. However, catalytic CHX might...
Also detoxify LPS [59]. Therefore, CHX has many faces but is still considered as “the gold standard of oral treatment”.

Epilogue

Extensive studies have been performed to explain the possible mechanisms involved in the pathogenesis of septic shock [12,5], chronic inflammatory processes such as seen in tuberculosis, arthritis, microbial- and fungal infections, periodontal disease and pulpitis, which many of them are also characterized by long-lasting granuloma formation [15,38,43-49]. Since cell and tissue damage in post-infectious sequelae is probably an end-result of “cross-talks” among a multiplicity of pro-inflammatory agents and the immune responses of the host [7-10,12], it may suggest that multi-drug strategies, but not single antagonists, could prove more effective protectors against the aftermath of chronic inflammatory episodes [16]. Bacteriolysis is a process where cationic agents such as lysozyme, cationic peptides and certain antibiotics, interfere with intracellular autolytic wall enzymes to induce cell lysis and the release of potent pro-inflammatory entities such as LPS, LTA and PPG [15] (Figure 1). The failure of phagocytes and their hydrolases to effectively dispose of microbial cell-wall components, (peptidoglycans) may be the hallmark of chronic granulomatous tissue responses seen in various inflammatory manifestations [15]. Reduction of tissue damage induced by LPS from Gram-negatives and by polycations from neutrophils may be achieved by sulfated polyanions and agents neutralizing LTA (phospholipids) from Gram positives might mitigate cell damage. The use of antibiotics in sepsis should be selected with care since these may also induce bacteriolysis and the release of highly phlogistic toxic microbial agents [27-29]. The role played not only by viable microorganisms but also by remnants of non-biodegradable cell-walls in chronic inflammatory episodes is appealing and looking for muramic acids, proteinases and cytokines (an overview). Free Radic Res. 1995; 22(6): 489-517.

Finally, the successful use of Chlorhexidine (CHX) in oral therapy which is due to its substantivity and relatively low toxicity is remarkable [68,69]. However, CHX use in the oral cavity might also act as a “double-edged sword” since it can also increase the consumption of H2O2 by catalase-rich commensals such as Candida albicans, E.coli, Staphylococcus aureus, Pseudomonas species and also by catalase-rich red blood cells endangering the survival of catalase-negative anaerobes, which are highly sensitive to H2O2 (Ginsburg et al., Submitted for publication).

Taken together, the dilemma whether cationic peptides and antibiotics-induced bacteriolysis in vivo is friends or a foe (a “double-edged swords?”) is still not clear [70]. However, the fact that only a very few new publications on bacteriolysis [71-73] had recently been published since we compiled our extensive review in 2002 [15], should not deter further new efforts to analyze and also perhaps “revive” the possible role of “the Focal infection hypothesis” as related to systemic manifestations.

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