Is Tissue Damage caused in Group A Streptococcal Infections Augmented by Synergizing with Neutrophils' Pro-inflammatory Products?

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ABSTRACT

Catalase-negative penicillin-sensitive group A hemolytic streptococci (GAS) are multifactorial microorganisms, which do not produce a unique damage-associated molecular patterns which if effectively neutralized might effectively stop their pathogenicity. GAS is involved in the pathogenicity of pharyngitis, tonsillitis, rheumatic fever, arthritis, necrotizing fasciitis (NF), toxic shock syndrome and also in sepsis. GAS-induced NF is quite a rare but dangerous and deadly infection, which most commonly occurs in the arms, legs and abdominal wall and is fatal in 30%-40% of cases. GAS, which possess surface capsular polysaccharide and antigenic M and T proteins, arrive at the inflammatory areas by generating spreading factors such as hyaluronidase, DNase and streptokinase-activated plasmin. GAS can spread in tissues and avidly adhere to membranes of target cells to deliver a non-immunogenic cell bound hemolysin (CBH) upon cells' membrane phospholipids to induce a penetrating membrane damage (“a kiss of Death”). Two additional potent extracellular hemolysins, Streptolysin O (SLO) and a non-immunogenic streptolysin S (SLS) produced can injure neutrophils (PMNs), which are recruited to the infected sites in large numbers. However, PMNs can engage in phagocytosis and also undergo activation to release various pro-inflammatory agents including NADPH-generated superoxide which dismutates to H₂O₂ and with myeloperoxidase (MPO) which forms toxic HOCl upon interaction with halides. Activated PMNs also deliver highly cationic peptides such as LL37, cationic elastase, cathepsins and nuclear histone, which interact electrostatically with negatively-charged membrane sites forming membrane lesions. PMNs also secrete many acid hydrolases, several Th1 cytokines and chemokines, which recruit more PMNs. Similarly, to beta-lactams antibiotics, cationic peptides can also activate bacteriolysis and trigger the release of the pro-inflammatory agents lipoteichoic acid (LTA) and peptidoglycan (PG).

We hereby propose that in infectious and inflammatory sites GAS and PMNs exo-products and also microbial cell-wall structures might all act synergistically to cause cell and tissue damage. Cell damage might be ameliorated by appropriate cocktails of anti-inflammatory agents. also, containing highly negatively charged heparin 23.

Keywords
Streptococcal infections, Microorganisms, Mammalian cells.

GAS Infections
The most studied clinical Infections caused in humans by catalase-negative group A hemolytic streptococci (GAS) are linked to pharyngitis, tonsillitis, rheumatic fever, rheumatic heart disease, arthritis, Saint Vitus’ dance, necrotizing fasciitis (NF), toxic shock syndrome, sepsis and organ failure [1-9]. Strains of group A streptococci isolated from patients with invasive necrotizing disease have been predominantly M types 1 and 3 that produce pyrogenic exotoxin A or B or both [7].

Recruitment of PMNs
GAS is characterized by their ability recruit huge numbers of PMNs to the infected sites. Scattered among damaged PMNs and host cells, histological section obtained from GAS tonsillitis show huge numbers of both intact and also demised host cells due to streptococcal hmolysins. While such activated PMNs may be cardinal for the killing of the invading GAS, they also have...
the ability to cause cell damage induced by the massive release of highly-toxic reactive oxygen and nitrogen species, cationic histones released from nets, LL37, cationic elastase, PLA₂, lysophosphatidies, and several TH1 cytokines.

What endows GAS their ability to injure mammalian cells?
During growth, GAS can generate a surface anti-phagocytic capsular polysaccharide and a highly immunogenic anti-phagocytic M proteins and many extracellular pro-inflammatory agonists [1-9].

These include: streptokinase, hyaluronidaseRNases, DNase, a cysteine proteinase, the extracellular hemolysin streptolysin O (SLO), the non-immunogenic cell-bound streptolysin S (CBH), which when bound to targets induces a “kiss of death”, the extracellular SLS, which binds to albumin, the cationic antimicrobial peptides (CAMPs), a complement inhibitor, supertoxin which dismutates to H₂O₂ and immunoglobulin degrading enzymes. SpeB, Which opposes PMNs migration, and superantigens Infective sites may also show the presence of GAS membrane-associated lipoteichoic acid (LTA) and cell-wall peptidoglycan (PPG) released following bacteriolysis induced either by antibiotics or also by various polycationic agents [10].

Human neutrophils (PMNs)
Activated human PMNs elaborate superoxide (generated via NADPH oxidase), SOD (superoxide which dismutates to H₂O₂), myeloperoxidase (MPO) which is important in netosis and in the release of the highly-toxic nuclear histone [11] possessing antibacterial and cytotoxic activities. H₂O₂ and a halide forming toxic HOCl, and nitric oxide (NO) synthase generates highly toxic peroxynitrite; the bactericidal and cytotoxic polycations LL-37, permeability inducing agents cathelicidin, cationic elastase, cathepsin G, gelatinase, several acid hydrolases, PLA2 and TH1 cytokines, which recruit PMNs [11-13]. Thus, during infection, mammalian tissues are simultaneously exposed to numerous cell injuring agents, which can either act singly or mainly in synergy to injure cells.

The synergism concept of cell damage in inflammation and infection
The notion that a ‘cross-talk’ among streptococcal toxins and proteinases and also among host-derived oxidants and several of the membrane-damaging proteinases generated in infectious and inflammatory foci originated back in 1959 [9]. It showed that catalase-negative group A hemolytic streptococci, which produced H₂O₂ and which also expressed on their surfaces the potent cell-bound membrane perforator streptolysin S (SLS), collaborated in a synergistic manner with a streptococcal serine proteinase to injure and disintegrate Ehrlich ascites tumor cells, a typical synergistic system [8]. Similarly, a synergistic cell injury also takes place between streptolysin O (SLO) and a streptococcal proteinase. These findings have also been fully corroborated by showing a synergy between streptolysin O (SLO) and a streptococcal proteinase in cell injury [8]. These observations established the basis for the “synergism concept of cellular injury in infectious inflammatory and in post-inflammatoryary process such as septic shock”. It is also hypothesizes that gangrene-inducing clostridial species and additional toxigenic bacteria can also act in synergy with activated PMNs to injure tissues. Bacteria and phagocytes possess adhesion molecules; they elaborate spreading enzymes (hyaluronidase, proteinases, nucleases), which facilitate their migration through the extracellular matrix and also secrete membrane-perforating cationic peptides, membrane active toxins and acid hydrolases. But above all, these pro-inflammatory agents seem to inflict a deadly blow on cells by a synergistic process. Hemolytic streptococci also share common antigens with the human heart, kidney, connective tissue and brain (molecular mimicry) which might explain some of the autoimmune features of post-streptococcal sequelae and rheumatic fever (RF) [6]. We therefore offer a general paradigm suggesting that tissue damage induced by GAS and by additional toxigenic and invasive bacteria is caused mainly by a synergism (cross talk) among their secreted agonists. Tissue damage can be further enhanced by PMNs secreted agents, by the microbial cell-wall products lipoteichoic acid (LTA) and peptidoglycan (PPG) released by cationic peptides and by bacteriolicytics [10]. Experimentally, it was shown that if human umbilical cord endothelial and epithelial cells in culture are mixed together with GAS and PMNs products, these may kill targets much faster and more efficiently by a synergistic process [14-21]. In GAS NF, there is also a close talk with GAS cysteine proteinase [19]. However, in NF caused by strains of GAS, toxic cationic histone are also released from flor necrotic tissues. Similarly, tissue cultures of endothelial cells treated by LL37, various oxidants, elastase, cathepsins, and histones generated by activated PMNs adhering upon endothelial cell are readily killed [22] (Figure 1).

However, the synergism phenomenon of tissue damage described may not be restricted only to GAS infection. It is reasonable to consider that as a rule of the thumb, microbial species, which recruit large numbers of PMNs to infectious sites, undergo necrosis by microbial toxins and participate cooperatively in cell damage. In this category, we may also include infections caused by Staphylococci (Figure 1), Pneumococci, Pseudomonas, Meningococci, Gonococci, in Crohn’s disease, Ulcerative Colitis, Periodontal disease, Urinary tract infections and in additional gastrointestinal disorders (to be published).

Can Use of cocktails of anti-inflammatory agents be effective to treat multifactorial infectious and post-inflammatory disorders?
It is proposed that being a multifactorial synergistic processes, it is recommended that in GAS infections multi drug strategies instilled into the infectious areas might prove effective [23]. This is in view of the failure over the years to treat sepsis by single antagonists [9]. Such cocktails may include non-bacteriolytic beta lactams, vancomycin, clindamycin, daptomycin, antioxidants such as N-acetyl cysteine, glutathione, polyphenols from green tea, pomegranate extract, coffee extract, IgG rich in anti-GAS antibodies, the poly anion heparin and even better, a non-anticoagulant heparin or polyanethole sulfonate. The polyanions may act by neutralizing the toxic effects of the highly-cationic
histone, LL-37, elastase and cathepsin G released from PMNs, and also histones released from necrotized tissues. Phospholipids may neutralize the microbial leukocidal hemolysins. SLO and SLS and PLA 2. Such cocktails can be easily concocted in any hospital pharmacy [16,23].

**Antibiotics treatment may release toxic microbial cell-wall components**

Especially in beta-lactam-treated GAS sensitive to penicillin, it may also be expected to locate in the inflamed tissues the microbial-derived capsular polysaccharides from GAS, peptidoglycan (PPG) and lipoteichoic acid (LTA) released from the Gram-positive bacteria following bacteriolysis [6]. Also, endotoxin (LPS) from mixed infections may also be released after treatment by antibiotics [10]. LTA released from GAS [24,25] was shown to avidly bind to surfaces of PMNs, to interact with anti-LTA antibodies present in nearly every normal patients serum resulting in the production of large amounts of superoxide, $\text{H}_2\text{O}_2$ and also to the release of lysosomal enzymes [25].

However, PPG-polysaccharide complexes released from GAS also possess potent pro-inflammatory properties causing chronic inflammation (see section below). Although *in vitro*, GAS is relatively resistant to degradation either by leukocyte extracts or by a cocktail containing lysozyme and lyso lecithin, they can become susceptible to lysis by leukocyte extracts if grown in the presence of sub-inhibitory amounts of penicillin (0.004-0.008μg/ml). Bacteriolysis becomes even more pronounced when the reaction mixtures are incubated at 41°C [26].

**Pathological changes induced by GAS cell-wall components**

Since certain beta-lactams and PMNs-derived cationic peptides can activate microbial autolytic wall enzymes also in GAS and to release toxic cell-wall components [10], we hereby present several of the numerous excellent pioneering investigations published by JH Schwab et al at the University of North Carolina [27-30]. The authors described the pathological changes induced mainly in joints, heart and liver of animals induced by complexes of PPG and polysaccharides derived from GAS. Such cell-wall components are resistant to biodegradation and can thus persist mainly in macrophage in inflammatory areas to cause chronic tissue damage. To prove that granulomas contain GAS products, Schwab and co-workers recommended to detect in the inflamed sites muramic acid, a major component of PPG which is an agent totally absent in mammals [28,30]. This elegant technique can also be employed on tissues derived from NF lesions.

Taken together, the failure to bio-degrade *in vivo* the cell-wall
complexes of GAS is an important phenomenon, which may explain the chronicity and the destructive process seen in various granulomatous processes. However, it should also be considered that many microbial species also developed certain agents, which may interfere with and retard PMNs activities (see section I), which may be balanced to some extent tissue damage induced during infections.

**Modulators of PMNs responses affecting GAS activity**

At least six major strategies in many microbial species had been identified suggesting how pathogenic bacteria and fungi use strategies to evade neutrophil defenses [31]:

- Turning on survival and stress responses
  - Avoiding contact
  - Preventing phagocytosis
  - Surviving intracellularly
  - Inducing cell death
  - Evading killing by neutrophil extracellular traps

In Streptococcal infections several factors can retard but not totally eliminate the toxic agents released. SpyCEP is a *Streptococcus pyogenes* protease that cleaves CXCL8/IL-8 and its activity is associated with human invasive disease severity [32]. SpyCEP cleaved human CXCL1, 2, 6 and 8 plus murine CXCL1. This protease is necessary and sufficient for systemic bacterial dissemination from a soft tissue focus in this model and also underlies dissemination in the respiratory tract protease SpyCEP (also called ScpC), which also cleaves IL-8. SpyCEP expression is strongly up-regulated *in vivo* in the M1T1 GAS strains associated with life-threatening.

Heparan Sulfate can modulate neutrophil and endothelial function in antibacterial innate immunity [33]. Normal bactericidal activity of neutrophils is influenced by the sulfation pattern of heparan sulfate by affecting traps formation. Still, this did not affect either phagocytosis or the formation of oxidants.

Platelets have been reported to form heterotypic aggregates with leukocytes and may modulate their function [34]. The endogenous platelet activator thrombin gave rise to platelet-dependent neutrophil activation, resulting in enhanced phagocytosis and bacterial killing. M1 protein from *Streptococcus pyogenes* also mediated platelet-neutrophil complex formation. However, these neutrophils were dysfunctional and exhibited diminished chemotactic ability and bacterial killing.

SLO in PMNs paralysis [35]. The secreted GAS pore-forming toxin streptolysin O (SLO), which induces eukaryotic cell lysis in a cholesterol-dependent manner, is highly up regulated in the GAS M1T1 clone during bloodstream dissemination. SLO promotes GAS resistance to phagocytic clearance by neutrophils, a critical first element of host defense against invasive bacterial infection. SLO at sub-cytotoxic concentrations suppressed neutrophil oxidative burst, in a manner reversed by free cholesterol and anti-SLO blocking antibodies. SLO blocked neutrophil degranulation, interleukin-8 secretion and responsiveness, and elaboration of DNA-based neutrophil extracellular traps, cumulatively supporting a key role for SLO as a modulator of PMNs’ functions.

SLS inhibits neutrophil recruitment [36]. In contrast to wild-type *S. pyogenes*, an SLS-mutant was associated with the robust recruitment of neutrophils and significantly reduced lethal myositis in adult zebrafish. Analysis of trans-epithelial migration *in vitro* suggested that SLS inhibited the host cells’ production of signals chemotactic for neutrophils, which contrasted with the pro-inflammatory effect of an un-related cytolytic toxin, streptolysin O. Whereas bacterial production of SLO resulted in lysis of both human keratinocytes and polymorphonuclear leukocytes, GAS expression of SLS was associated only with keratinocyte injury. Expression of SLO but not SLS impaired polymorphonuclear leukocyte killing of GAS *in vitro*, but this effect could only be demonstrated in the background of capsular organisms. To facilitate invasion, *S. pyogenes* secrete streptokinase (SK), a potent plasminogen activator. SK-plasminogen interactions are important determinants of GAS invasiveness *in vivo* and both SK and host plasminogen activators appears to promote virulence of GAS by catalyzing plasmin formation and produce high rates of morbidity and mortality despite the implementation of aggressive treatment plans.

DNase activity made by GAS contributes to disease progression by affecting degradation of neutrophil extracellular traps. These innate immune structures are composed of chromatin and granule proteins [37].

Incompetence of neutrophils to invasive group A streptococci [38]. A panel of serotype-matched GAS, which were clinically isolated from severe invasive but not from non-invasive infections, could abrogate functions of human polymorphonuclear neutrophils (PMN) in at least two independent ways; due to inducing necrosis to PMN by enhanced production of a pore-forming toxin streptolysin O (SLO) and due to impairment of PMN migration via digesting interleukin-8, a PMN attracting chemokine, by increased production of a serine protease SepC.

Chemotactic factor [39]. Human polymorphonuclear leukocytes (PMN) chemotaxis was tested during exposure to leukocyte and platelet extracts, a variety of polyelectrolytes, inflammatory exudates and bacterial products. The chemo-attractants employed were eitherzymosan-activated serum or supernatant from autolyzed *Staphylococcus aureus*. Chemotaxis to both chemo-attractants was markedly inhibited by leukocyte and platelet extracts, inflammatory exudates, anionic polyelectrolytes, DNA, hyaluronic acid, liquid and by cationic polyelectrolytes, histone, protamine base, protamine sulfate, and myeloperoxidase, elastase, collagenase, pepstatin, and epsilon-aminocaproic acid. Bacterial products, such as lipoteichoic acid and lipopolysaccharides, and extracts of human dental plaque inhibited chemotaxis.

**Conclusion**

The purpose of the present treatise is to offer a reasonable paradigm, which suggests that cell and tissue damage in GAS-
infections, as well as in additional microbial infections and inflammation, may not be induced solely by their direct toxic attack but in a tight synergy with numerous pro-inflammatory products generated by the myriads of PMNs recruited to the inflammatory sites creating a vicious cycle. Actually, we could not find a clear-cut explanation what are the recruited PMNs really doing upon arrival at the inflammatory areas. Can we postulate that PMN tasks are to intercept with, to engulf and to kill GAS? or is it not more reasonable to assume an additional scenario? This is when PMNs armed to teeth with oxidants, proteinases, cationic peptides, histone, PLA₂ and additional lysosomal hydrolases aided by histones released from necrotic tissues may all converge, in a synergistic manner, with the microbial pro-inflammatory ex-products and with their cell-wall components lipoteichoic acid (LTA) and peptidoglycan (PPG) to destroy the tissues. This assumption was supported by large numbers of in vitro studies using human umbilical cord endothelial cells and epithelial cells in culture [14-21]. These mainly showed combined effects among oxidants, proteolytic enzymes and cationic peptides. This was quantified in many studies by measuring the release from the target cells of radio-labeled membrane associated arachidonic acid. However, this may perhaps also be verified by squeezing out fluids from necrotic areas to be tested for toxicity to cultures and also to show presence of myeloperoxidase, a typical marker of PMNs [11,12] and also measure muramic acid, the integral component of the microbial cell-walls. Although GAS can generate various toxic agents, the endless waves of fresh PMNs recruited to the infectious sites may overcome the microbial toxic action. To overcome the toxic effects exerted by the synergism between bacterial and PMNs agonists, it may be recommended to infuse into the morbid tissues a cocktail of appropriate antagonists. These may be comprised of combinations among non-bacteriolytic clindamycin, daptomycin, or linezolid, antioxidants from green tea and from pomegranate, proteinases inhibitors, anionic heparin, phospholipids, pooled immune globulins and also antibodies to selected anti-TH1 cytokines [16,40]. These may also be encased in liposomes or other drug delivery platforms to secure a better absorption and bioavailability. Such cocktails may neutralize to some extent the synergies among the toxic agents generated by the combinations of bacterial and PMNs pro-inflammatory agents. However, we should not under estimate that the arrival of PMNs at the inflammatory sites may eventually also have important functions in the final resolution of inflammation and restoration of normal tissues lost by the destructive effects of the inflammatory process. Unfortunately, healing the tissue in NF may not happen too often. Therefore, it should be decided quickly how to proceed with treatment since the grarest danger to patients’ lives suffering of NF is its high invasiveness and destructiveness leading to high morality. Therefore, to save patients’ lives necessitates a quick radical surgery and debridement of necrotic tissues. Finally, since many additional severe microbial infections characterized by a massive recruitment of PMNs are also defined as multifactorial and synergistic episodes, the use of antibiotics combined with cocktails of antagonists may also be helpful to contain the infections and their aftermath. Finally, we have also postulated that hemolytic streptococci may be some kind of “fore fathers of modern PMNs [41].

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