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Multi-drug strategies are necessary to inhibit the synergistic mechanism causing tissue damage and organ failure in post infectious sequelae

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Abstract—The paper discusses the principal evidence that supports the concept that cell and tissue injury in infectious and post-infectious and inflammatory sequelae might involve a deleterious synergistic interaction among microbial- and host-derived pro-inflammatory agonists. Experimental models had proposed that a rapid cell and tissue injury might be induced by combinations among subtoxic amounts of three major groups of agonists generated both by microorganisms and by the host's own defense systems. These include: (1) oxidants: superoxide, H_2O_2, $OH^{\cdot}$, oxidants generated by xanthine-xanthine-oxidase, $ROO^{\cdot}$, HOCl, NO, $OONO^{\cdot}-$, (2) the membrane-injuring and perforating agents, microbial hemolysins, phospholipases A_2 and C, lysophosphatides, bactericidal cationic proteins, fatty acids, bile salts and the attack complex of complement a, certain xenobics and (3) the highly cationic proteinases, elastase and cathepsin G, as well as collagenase, plasmin, trypsin and a variety of microbial proteinases. Cell killing by combinations among the various agonists also results in the release of membrane-associated arachidonate and metabolites. Cell damage might be further enhanced by certain cytokines either acting directly on targets or through their capacity to prime phagocytes to generate excessive amounts of oxidants. The microbial cell wall components, lipoteichoic acid (LTA), lipopolysaccharides (LPS) and peptidoglycan (PPG), released following bacteriolysis, induced either by cationic proteins from neutrophils and eosinophils or by beta lactam antibiotics, are potent activators of macrophages which can release oxidants, cytolytic cytokines and NO. The microbial cell wall components can also activate the cascades of coagulation, complement and fibrinolysis. All these cascades might further synergize with microbial toxins and metabolites and with phagocyte-derived agonsits to amplify tissue damage and to induce septic shock, multiple organ failure, 'flesh-eating' syndromes, etc. The long persistence of non-biodegradable bacterial cell wall components within activated macrophages in granulomatous inflammation might be the result of the inactivation by oxidants and proteinases of bacterial autolytic wall enzymes (muramidases).

The unsuccessful attempts in recent clinical trials to prevent septic shock by the administration of single antagonists is disconcerting. It does suggest however that, since tissue damage in post-infectious syndromes is most probably the end result of synergistic interactions among a multiplicity of agents, only agents which might depress bacteriolysis *in vivo* and 'cocktails' of appropriate

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antagonists, but not single antagonists, if administered at the early phases of infection especially to patients at high risk, might help to control the development of post-infectious syndromes. However, the use of adequate predictive markers for sepsis and other post-infectious complications is highly desirable. Although it is conceivable that anti-inflammatory strategies might also be counter-productive as they might act as 'double-edge swords', intensive investigations to devise combination therapies are warranted. The present review also lists the major anti-inflammatory agents and strategies and combinations among them which have been proposed in the last few years for clinical treatments of sepsis and other post-infectious complications.

Key words: Inflammation; infection; synergism; bacteriolysis; sepsis; organ failure; anti-inflammatories.

1. INTRODUCTION

It is disconcerting and alarming that approaching the third millennium clinicians are still bewildered and too often helpless when trying to cope with the life-threatening sequelae of severe microbial infections. Despite recent impressive advances in the development of novel and sophisticated immunological and molecular–biologlogical technologies and strategies (Wheeler and Barnard, 1996; Horn *et al.*, 1996; Luckacks and Ward, 1996; Pinsky, 1996; Hack *et al.*, 1997; Liu and Slutzki, 1997; Schlag and Redl, 1999; Vincent, 1997; Baue, 1998; Baue *et al.*, 1998; Faist and Kim, 1998; Furies, 1998; Yao *et al.*, 1998) and the aggressive use of antibiotics, the mortality due to septic shock, ARDS and the 'flesh-eating' microbial syndromes, is still very high. It is very discouraging that no less than 29 prospective controlled studies of human sepsis that have been conducted during this decade and which had mostly tested the efficacy either of one single, non-steroidal or steroidal anti-inflammatory drug, failed to show a significant protection against the morbidity and lethality of septic patients (Natanson, 1997; Schlag and Redl, 1999; Baue, 1998; Opal and Liu, 1998; Nasraway, 1999; Wheeler and Bernard, 1999). Several reports have also warned that combination therapies might even enhance mortality (Baue, 1998; Opal and Liu, 1998). Why have these therapeutic strategies invariably failed to cope with the sequelae of severe microbial infections and what future approaches might break the stalemate leading to a better understanding of the pathophysiology of the 'horror autotoxicus' phenomena (Baue, 1992, 1998) emerging from the sequelae of the invasion of the blood stream either by Gram-negative (Yao *et al.*, 1998), or by Gram-positive bacteria (Stevens, 1995)? The inability to offer adequate therapeutic measures to treat post-infectious sequelae has recently led to the publication of a plethora of controversial articles, letters to the editor and view points attempting to explain these failures. It has also been suggested that clinicians and basic scientists should get together, go back to the drawing board, and propose novel approaches of therapies (Verhoef *et al.*, 1996). It has also been questioned recently whether the continuation of clinical trials with only a marginal benefit, is ethical (Nasraway, 1999). This pessimistic stand might stem from the realization that no single omnipotent pro-inflammatory agonist exists, which if effectively ad-

ministered might avert the complex synergistic interactions among a multiplicity of agonists responsible for the initiation of cell and tissue injury (Ginsburg and Kohen, 1995; Ginsburg, 1998; Ginsburg and Sadovnic, 1998; Ginsburg *et al.*, 1999; Baue, 1998; Wheeler and Bernard, 1996).

The purpose of this viewpoint is to stress the roles of uncontrolled bacteriolysis and of a multiplicity of pro-inflammatory agonists functioning in concert (synergism) to induce tissue damage and organ failure in post-infectious sequelae, and also to speculate that only 'cocktails' of antagonists might have a beneficial effect. These should be administered preferentially, as prophylactics, to high-risk patients, and to all the patients showing very early signs of an invasion of the blood stream by microorganisms. This however, depends on the availability of inexpensive, rapid and reliable predictive tests, at the bedside, to herald the early development of post-infectious complications (see below).

2. THE SYNERGISM CONCEPT OF CELLULAR INJURY

We need a realistic explanation of how tissues are destroyed and organs fail in inflammatory, infectious and in post-infectious manifestations. The concept that tissue damage initiated during and following microbial invasion, might be caused by interaction between several pro-inflammatory agonists, had emerged form observations on the pathophysiology of tissue damage induced by catalase-negative bacteria (streptococci, clostridiae) (Ginsburg, 1972, 1996, 1998, 1999; Ginsburg *et al.*, 1995, 1998; Ginsburg and Sadovnic, 1998). Already by 1959, it had been shown that tumour cells injured by the membrane-perforating toxin, steptolysin S (SLS), were rapidly disintegrated by non-cytolytic amounts of a cysteine proteinase derived from streptococci (Ginsburg, 1972). Other studies from our laboratory (see Ginsburg and Kohen, 1995) had also shown that tumour cells injured by complement-dependent cytotoxic antibodies were rapidly disintegrated by sub-toxic amounts of streptokinase-activated plasmin suggesting that a membrane injury altered the plasma membrane to facilitate a subsequent protease attack. Since the catalase-negative streptococcus also produces large amounts of H_2O_2 during growth, we postulated that a membrane injury by a perforator might synergize with peroxide and with proteinases to overcome the potent antioxidant capacities of mammalian cells (Ginsburg, 1994; Ginsburg and Kohen, 1995). Furthermore, it is highly probable that, *in vivo*, combinations among microbial-derived membrane perforating toxins, phospholipases, oxidants, proteinases, hyaluronidase, DNAse, RNAse, neuraminidase, chondroitin sulphatases and heparinase, might explain the capacity of streptococci and the gangrene-producing Clostridiae to disseminate to remote tissue sites where they can attack target cells by synergism among a multiplicity of agonists. Since both streptococci and several of the clostridial species (gas-gangrene producers), mighty mimic activated phagocytes (neutrophils, eosinophils, macrophages) which are also known to generate a plethora of strikingly similar pro-inflammatory agonists (Ginsburg, 1994, 1999; Ginsburg and Kohen, 1995; Stevens,

1995) it was postulated that in infectious sites, microbial and phagocyte-derived agonists might also engage in a synergistic interaction to amplify tissue damage.

The concept that a synergistic mechanism among a multiplicity of agonists might be central in cell damage was also examined in a number of laboratories. These investigated the synergistic killing of radiolabelled mammalian cells, in culture (endothelial cells, epithelial cells, fibroblasts, tumour cells) and the destruction of extra cellular matrix proteins, initiated by combinations among a large variety of agonists. These included oxidants (H_2O_2, $OH^{\cdot}$ NO, $ROO^{\cdot}$, HOCl), membrane perforating agents (the streptococcal hemolysins S and O, phospholipases A_2 and C, lysophosphatides, fatty acids, cationic proteins, certain xenobiotics, the attack complex of complement), proteinases (plasmin, elastase, cathepsin G, trypsin, pseudomonas- and streptococcal-derived proteinases) (Baird *et al.*, 1986; Weiss *et al.*, 1986; McGowan and Murry, 1987; Weiss, 1987; Lichtenstein *et al.*, 1988; Mendis *et al.*, 1990; Klebanoff *et al.*, 1983; Ginsburg and Kohen, 1995; Ginsburg, 1994, 1998, 1999; Fujiata *et al.*, 1996; Zanetti and Glauser, 1997), and cytokines (Ferrante *et al.*, 1992).

It was demonstrated that irrespective of the sources of agonists tested, whether of microbial or of host-origins, a triad consisting of a *membrane perforator*, an *oxidant* and a *proteinase* constituted a potent cell killing cocktail which also induced the release of large amounts of arachidonate and its metabolites as well as additional fatty acids. It is also of great interest that the cytolytic effects induced by combinations among perforators, oxidants and proteinases can be markedly depressed either by inhibiting the perforator or of the oxidant suggesting that the simultaneous presence of these two agents is absolutely required to induce an irreversible membrane injury (Ginsburg and Sadovnic, 1998). These studies implicate that paradoxically, perhaps, during microbial infections there might occur a deleterious synergistic interaction among microbial and host-derived agonists.

The mechanisms by which combinations of oxidants, membrane-perforators and proteinases injure cells might involve a removal, by oxidants and perhaps also by proteinases, of membrane-associated glycosaminoglycans which facilitated the exposure the 'naked' plasma membrane to an attack by the membrane-perforating agents (Dan *et al.*, 1996).

Furthermore, it might also be considered that LPS, lipoteichoic add (LTA), peptidoglycan and toxic shock syndrome toxins (super antigens) might be released by microorganisms either during growth or following bacteriolysis induced either by neutrophil-derived polycations and cationic proteinases (Ginsburg, 1987, 1988)or by beta-lactams (Holzheimer, 1998, Periti and Mazzeti, 1998). These might activate mononuclear cells to generate reactive oxygen and nitrogen species, hydrolases, spreading enzymes, cytotoxic cytokines (TNF-α INF-γ and interleukins (IL)-1, IL-2, IL-6) and also to trigger the activation of the coagulation and complement cascades (Baue, 1998; Baue *et al.*, 1998; Schlag and Redl, 1999). Also, oxidants and proteinases released into the phagolysosomes of phagocytes might inactivate the autolytic wall enzymes in autolytic bacteria (Ginsburg, 1989) contributing

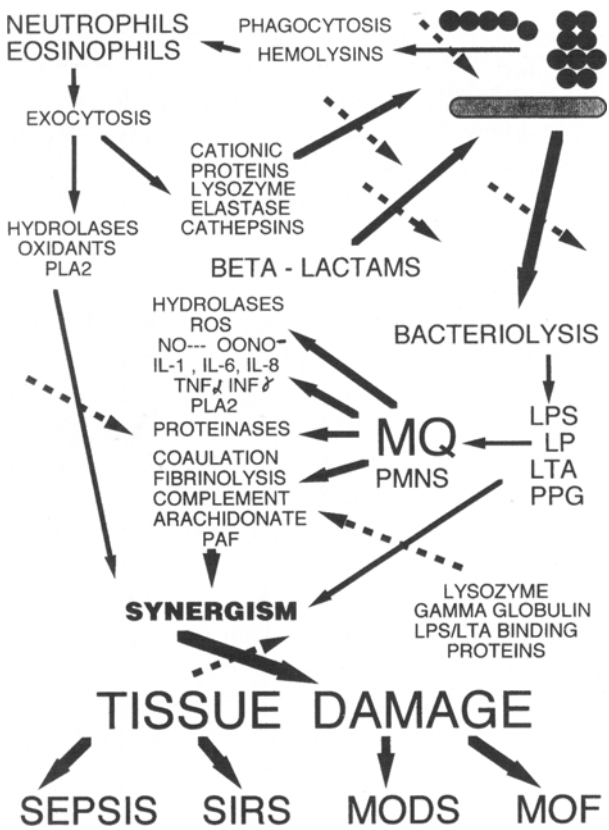

Figure 1. Gram-positive and Gram-negative bacteria which elaborate membrane-damaging hemolysins might interact with *neutrophils* and *eosinophils* resulting in phagocytosis, a release of reactive oxygen species and exocytosis of lysosomal hydrolases, cationic proteins, and the highly cationic proteinases (lysozyme, elastase, cathepsin G) and collagenase. Cationic proteins, beta-lactams and additional bacteriolytic antibiotics might induce cell wall degradation and the release of lipoteichoic acid (LTA), lipopolysaccharide (LPS), lipoprotein (an LPS mimic) and peptidoglycan (PPG). These might interact with mononuclear phagocytes macrophages-MQ to induce the generation and release of reactive oxygen species. Generation of NO might lead to the formation of the highly cytotoxic peroxinitrite (OONO-). Neutrophils and nacrophages might also release acid hydrolases, PLA_2, cytotoxic cytokines (INF-γ, TNF-α, IL-2 and IL-6) and also trigger the coagulation, complement, arachidonate and PAF cascades. Many of the agents released by microorganisms, by activated phagocytes by platelets and by the host's humoral systems, might act in synergy to amplify cell and tissue damage which might culminate in septic shock, SIRS, MODS and MOF. The dashed arrows indicate where inhibitory agents and cocktails of antagonists might attenuate tissue damage.

to the persistence of non-biodegradable microbial peptidoglycan-polysaccharide in granulomas (Ginsburg, 1972, 1979). It is therefore speculated that unless bacteriolysis is inhibited at the very early stages of infection (see Wecke *et al.*, 1987, 1990; Kiriyama *et al.*, 1987; Ginsburg, 1988; Periti and Mazzeti, 1998), microbial-derived toxins and cell-wall components might synergize with exo-products generated by activated phagocytes and with humoral immune processes,

to significantly amplify cell and tissue damage often leading to sepsis, septic shock, ARDS, fasciitis and myositis (the flesh-eating bacterial syndromes) (Stevens, 1995) and also in multiple organ failure. Figure 1 illustrates the main possible pathways of pathogenicity of tissue damage and the ensuing post-infectious sequelae resulting from infections with Gram-positive and Gram-negative bacteria.

3. POST-INFECTIONS SEQUELAE

The lack of adequate predictive markers for post-infectious sequelae is the Achilles heal of clinical practices. The difficulties in predicting the development of sepsis and its aftermath is undoubtedly the main concern to clinicians (Baue, 1998; Baue *et al.*, 1998; Wheeler and Bernard, 1999). Patients arriving at the emergency room and suspected of having early signs of post-infectious sequelae, might already be under aggressive antibiotic treatment. This might greatly delay the isolation and characterization of microorganisms in the blood stream and the beginning of appropriate antibacterial and anti-inflammatory treatments.

The identification and measurements of the levels of the following predictive markers in sera and in other body fluids, at the bedside, have been proposed to help in the early treatment of septic patients. These markers include: leukocyte counts and leukocyte-derived pyrogens, LPS, LTA, peptidoglycan, lysozyme, CRP and additional acute phase proteins, lactate, procalcitonin, antithromnin III, TNF-α INF-γ, IL-1, IL-6 additional cytotoxic cytokines, LPS binding proteins and LPS binding cationic proteins. A significant rise in many of these markers, especially of IL-6, have been documented even in the absence of a positive blood culture. Unfortunately, the present high costs of such tests is a major obstacle to any future proper medical practice.

3.1. Strategies to cope with post-infectious sequelae

The realization that tissue damage in infectious and post-infectious manifestations is the end result of multiple synergistic interactions among a multiplicity of pro-inflammatory agonists and that the host might also generate anti-inflammatory agents, is paradoxical, controversial and confusing (Baue, 1998; Wheeler and Bernard, 1999). Therefore, the dilemma whether it might be safe to expose the host to therapeutic regimens which might also depress anti-inflammatory agents, haunts clinicians. However, it is unrealistic to expect that an exclusive administration to patients either of super doses of anti-oxidants, anti-proteinases, inhibitors of LPS, LTA, TNF-α and of cytotoxic cytokines, anticoagulants etc., might be able to totally eradicate the bulk of the corresponding pro-inflammatory agonists generated in infectious sites.

Therefore, even the small but subtoxic amounts of agonists remaining might still synergize among themselves to injure cells and tissues (Ginsburg and Kohen, 1995; Ginsburg, 1998, 1999). This suggests that the early inhibition of bacteriolysis in the

blood stream, the resulting release of cell wall components (see below) and the use of combinations among several drugs directed against the most important microbial and host-derived agonists, might be the most effective measures to attenuate the synergistic cell-injuring cascades.

Screening of the literature on sepsis has revealed that no less than 32 (!) different therapeutic agents and strategies have been proposed and employed to protect against septic shock either in experimental animals or in the 29 (mostly unsuccessful) clinical trials of sepsis performed in this decade (Ralston and St. John, 1996; Pinsky, 1996; Liu and Slutzki, 1997, Opal and Liu, 1998; Triujillo *et al.*, 1998; Baue, 1998; Baue *et al.*, 1998; Schlag and Redl, 1999; Nasraway, 1999). These treatments included: antibiotics, monoclonal and polyclonal antibodies against LPS TNF- and cytokines, receptor antagonists, LPS-binding proteins, microbial permeability enhancing cationic peptides (BPI), polymyxin B, lysozyme, gamma globulin, proteinases inhibitors, azo dyes, lipids and phospholipids, prostacyclines, sulphated anti-coagulants, anti-thrombin III, plasminogen activatior inhibitor (PAI-1), scavengers of reactive oxygen and of nitrogen species, inhibitors of NO synthase, tyrosine kinase inhibitors, anandamites, pentoxyphilline, FAF antagonists, inhibitors of adhesion molecules, steroids and amino steroids NSAIDs, inhibitors of the nuclear factor NFkB, PLA_2 inhibitor, angiotensin converting enzymes (ACE), high volume haemofiltration techniques, lactulose, glucans, bradykinin and histamine antagonists, lactoferrin feeding and colony stimulating factors (GCSF, GMCSF), tetracyclines IL-10. Many of these agents had previously been proven effective in preventing shock and organ failure in small laboratory animals provided, however, that they had been administered before the injection either of LPS or the performance of caecal-ligation and puncture, a common method to induce shock and organ failure. This clearly indicates that once the deleterious biochemical and pharmacological cascades induced by microbial agents are activated; no singly administered antagonist might be effective if given too late. This further stresses the need for predictive markers in any future treatment of septic patients.

Based on the above mentioned, it might be postulated that only cocktails of carefully selected antagonists, when given on time, might cope with the complex synergistic interactions responsible for the initiation of shock and organ failure. Therefore, priority should be given to the future developments of agents capable of inhibiting bacteriolysis and the release of microbial cell-wall components.

The following agents and combinations among them might be considered: (1) non-bacteriolytic antibiotics (vancomycin in the cases of infections with Gram-positive bacteria and antibiotics which inhibit *de novo* synthesis of muramidases in the cases of Gram-negatives), (2) inhibitors of bacterial autolytic wall enzymes (the sulphated polysaccharides heparin, dextran sulphate, polyanethole sulphonate, Evan's blue (Wecke *et al.*, 1987, 1990; Ginsburg, 1987, 1988), D-amino acids (Tuomanen and Tomasz, 1984)) shown to inhibit polycation- and beta lactam-induced bacteriolysis. Polyanions might also inhibit disseminated intravascular coagulopathy (DIC), the attack complex of complement as well as the highly cytolytic cationic

proteins generated by neutrophils and eosinophils, (3) pooled gamma globulin to neutralize microbial exo-enzymes and toxins, (4) selective antibodies to LPS, TNF-α, cytotoxic cytokines and adhesion molecules, (5) antioxidants (N-acetyl cysteine, vitamin E, ebselen, melatonin, taurine, dimethylthiourea, allopurinol), (6) inhibitors of NO-synthase (N-monomethyl arginine) and of NO (methylene blue), (7) proteinase inhibitors (aprotinin, ε-amino caproic acid), (8) phospholipids, cholesterol and Evans' blue to counteract microbial hemolysins, and the attack complex of complement, (9) tetracyclines and pentoxyphilline to inhibit TNF-production, (10) tyrosine-kinase inhibitors and (11) anandamites, to mention only several of the agents which might be effective. It is of great interest that both tyrosine kinase inhibitors (Servansky *et al.*, 1997) and anandamites (Gallili *et al.*, 1997), have recently been shown to be also effective even if administered to animals after LPS. Also, Tibetan herbal preparations such as PADMA-28 were shown to possess potent anti-oxidant, antiproteinases (Ginsburg, 1999), anti-cytokine activities (Ginsburg and Barak, to be published) and also to be effective in the amelioration of the condition of atherosclerosis patients (Sallon *et al.*, 1998). This preparation may also be promising as an adjunct to counteract the synergistic cascades which develop during septic shock.

Undoubtedly, it is mandatory that animal models to test combination therapies to cope with synergistic mechanisms of tissue injury, in sepsis and in allied conditions be established prior to the formulation of any future safe cocktail therapies in humans and this in view of reports concerning the enhanced mortality of experimental animals induced by combination therapies (Natanson, 1997; Baue, 1998; Nasraway, 1999).

4. CONCLUSION

It is enigmatic why none of the numerous publications which have described the possible role played by polycations of leukocyte origins as potent activators of bacteriolysis, its possible inhibition, and the role played by synergistic interactions among a multiplicity of pro-inflammatory agonists in cellular injury, had never been discussed in any of the extensive literature on sepsis and septic shock. It is regrettable that this is how pioneering investigations so relevant to sepsis research are 'forgotten and most probably also buried for ever'. It is hoped, however, that a way might be found to bring these 'lost' publications and viewpoints to the attention of clinicians and to the pharmacentics industry as it might contribute to formulations of safer and more efficient magic bullets to control and prevent the aftermath of severe systemic infections in humans.

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