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Antibiotics as Anti-Inflammatory and Immunomodulatory Agents

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Editors

Birkhäuser Verlag
Basel · Boston · Berlin

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A CIP catalogue record for this book is available from the Library of Congress, Washington D.C., USA

Bibliographic information published by Die Deutsche Bibliothek
Die Deutsche Bibliothek lists this publication in the Deutsche Nationalbibliografie;
detailed bibliographic data is available in the internet at http://dnb.ddb.de

ISBN 3-7643-5925-0 Birkhäuser Verlag, Basel – Boston – Berlin

Part of Springer Science+Business Media
Printed on acid-free paper produced from chlorine-free pulp. TCF ∞
Cover design: Markus Etterich, Basel
Cover illustration: Inhibitory effect of clarithromycin on LPS-induced MAC5AC gene expression and I-kappa-B-alpha phosphorylation in human airway epithelial cells. With the friendly permission of Jun Tamaoki.
Printed in Germany
ISBN 3-7643-5925-0

9 8 7 6 5 4 3 2 1 www.birkhauser.ch

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Preface

The antibiotic era began in earnest during World War II with the "miracle of penicillin". Following the introduction of penicillin, the quest was on to discover similar antimicrobial agents. In the late 1940s, erythromycin A was isolated from a soil sample found in the Philippine island of Iloilo, and in 1952 erythromycin was introduced by Eli Lilly Company under the name of Ilosone, as an alternative to penicillin for emerging penicillin-resistance bacteria. It was recognized early on that the gastrointestinal side effects of erythromycin A could be modified by altering the chemical structure of the agent, and in the early 1990s clarithromycin and azithromycin were developed to be more acid-stable and with fewer side effects. Not long after this, it was shown that the macrolide antibiotics had immunomodulatory effects separate from antimicrobial properties.

The "steroid sparing" properties of the 14-member macrolides troleandomycin and oleandomycin, were first described in patients with severe, steroid-dependent asthma. Erythromycin was also found to reduce the need for corticosteroids in patients with asthma and, as described by Rose Jung, Mark H. Gotfried and Larry H. Danziger, in these trials some severe, steroid-dependent asthmatics were able to discontinue systemic corticosteroids with the use of macrolide antibiotics. Although it was speculated that the mechanism of macrolide action for severe asthma was by interfering with corticosteroids metabolism, in the clinical trials the reduction in steroid side effects, dosage, and in some cases discontinuation of steroids suggested a different effect on the underlying disease.

This was exploited in the 1980s in Japan for the treatment of the nearly uniformly fatal airway disease diffuse panbronchiolitis (DPB), as described by Arata Azuma and Shoji Kudoh. Since that time, many investigators in Japan – and now around the world – have studied these immunomodulatory properties not only of macrolide antibiotics but also of other classes of antimicrobials. Studies in the last 5 years have confirmed these effects, not only for the treatment of DPB but for also cystic fibrosis (CF) as discussed by Adam Jaffé and Andrew Bush. With the widespread adoption of macrolide therapy for the treatment of CF there has been an explosion of interest and publications in the field. A literature search conducted in

June 2004 from the PubMed database shows that there have been nearly 300 references to the immunomodulatory or anti-inflammatory properties of antibiotics since 1976.

This book is divided into two sections; the first, on basic research, evaluates the effects of macrolide antibiotics on bacteria other than by ribosomally-mediated bacteriostasis. Specifically the macrolide antibiotics have been shown to influence the expression of virulence factors in gram-negative organisms and decrease the ability of these bacteria to form biofilms as detailed in the chapters by Kazuhiro Tateda, Theodore J Standiford, and Keizo Yamaguchi. A series of six chapters then follow detailing the various anti-inflammatory and immunomodulary effects of these antibiotics. Immunomodulation in this sense refers to the ability to downregulate deleterious hyperimmunity leading to airway damage as opposed to anti-inflammatory properties, which refers to the suppression of all inflammatory responses whether beneficial or not. Thus immunomodulation should not impair the normal host defense but will prevent an acute inflammatory response from becoming chronic and destructive inflammation. Michael Parnham gives a superb overview of the role of inflammation and its resolution with antibiotics. This is then followed by chapters that document the effect of macrolide antibiotics on cell membrane protection and epithelial stabilization (Charles Feldman and Ronald Anderson), neutrophil activation and chemotaxis (Jun-ichi Kadota), reduction of proinflammatory cytokine expression and release (HajimeTakizawa), the oxidative burst (Marie-Thérèse Labro), and immune activation (Jun-ichi Kadota).

Related to these immunomodulatory effects are the effects on mucus secretion. It is well established that mucus secretion is beneficial to the airway preventing bacterial infection, airway desiccation, and aiding particle clearance; however mucus hypersecretion can lead to airflow obstruction and entrap microorganisms as seen in patients with chronic airway inflammation. Many chronic inflammatory airway diseases such as COPD, asthma, sinusitis, DPB, bronchiectasis and CF are associated with hyperinflammation and airway obstruction with secretions. Kiyoshi Takeyama discusses the role of macrolides in mucus production and secretion and Jun Tamaoki reviews the related data on the regulation of ion channels and how this relates to macrolide antibiotics and mucus secretion.

The second part of the book discusses the clinical results using antibiotics as mucoregulatory agents in a variety of diseases. Shoji Kudoh, who was the first to describe the role of macrolides in the treatment of DPB, and Arata Azuma provide a superbly updated overview of DPB including the current Japanese recommendations for the use of macrolides in treating this disease. These guidelines have proven useful for establishing appropriate therapy for Adam Jaffé and Andrew Bush, who discuss not only their landmark studies of azithromycin for the treatment of CF but also the results of recent large-scale studies that have led to wide acceptance of this therapy. This is followed by a chapter by Kazuhiko Takeuchi, Yuichi Majima, and Qutayba Hamid that reviews the use of macrolides in the therapy chronic upper air-

way diseases including sinusitis and nasal polyposis. Rose Jung, Mark H. Gotfried, and Larry H. Danziger then summarize the use of macrolides and the treatment of chronic asthma; in particular for persons with neutrophil-predominant, steroid dependent asthma. The role of immunomodulatory antibiotics in the treatment of lung injury is reviewed by Arata Azuma.

Eiji Kita, Keiichi Mikasa and Kei Kasahara give a superb review of the data suggesting a possible role of immunomodulatory antibiotics that can decrease proinflammatory cytokines for the therapy of nonpulmonary disorders including arthritis, inflammatory bowel disease, and cancer. The final chapter by Markus O. Henke, Axel Dalhoff, and Bruce K. Rubin reviews the immunomodulatory properties of antibiotics other than macrolides with the special emphasis on the quinolones, where data now support the ability of these agents to affect the immune systems.

This is an exciting and a rapidly changing field and we are delighted to have the opportunity to summarize the state of the art as of 2004. Thus it is timely that this book be published summarizing these data and it is appropriate that half of the authors are from Japan. We personally believe it is likely that we will see a more widespread use of these antibiotics for their immunomodulatory properties as well as the development of derivatives of these medications that have no antibacterial properties but that do have more potent and directed immunomodulatory activity. This may permit more precise therapy for preventing biofilm diseases or chronic inflammation while reducing the risk of developing antimicrobial resistance to the macrolide class of antibiotics. The editors would like to thank Michael Parnham, the PIR series editor, for suggesting this book and for agreeing to write the overview chapter. We would also like to thank our editors at Birkhäuser Publishing including Karin Neidhart and Hans Detlef Klüber for their outstanding support. Finally the Editors of this monograph would like to thankfully acknowledge the many students and postdoctoral investigators who have worked with us over the years and enriched both our research laboratories and our lives.

Winston-Salem/Tokyo, July 2004

Bruce K. Rubin
Jun Tamaoki

I. Basic research

Indirect antimicrobial effects

Effects of antibiotics on *Pseudomonas aeruginosa* virulence factors and quorum-sensing system

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes a wide range of acute and chronic infections, including sepsis, wound and pulmonary infections [1]. In particular, this organism is a major cause of pulmonary damage and mortality in patients with cystic fibrosis (CF), diffuse panbronchiolitis (DPB) and other forms of bronchiectasis [2, 3].

P. aeruginosa is known to produce a variety of virulence factors, such as pigment and exotoxins. The synthesis and expression of these factors is regulated by a cell-to-cell signaling mechanism referred to as quorum sensing [4, 5]. Two major quorum-sensing components in *P. aeruginosa*, *Las* and *Rhl*, enables bacteria to coordinately turn on and off genes in a density-dependent manner by the production of small diffusible molecules called autoinducers [6, 7]. The expression of these autoinducer-regulated virulence factors directly contributes to the course and outcome of individuals infected with *P. aeruginosa*.

A breakthrough in chemotherapy for patients with chronic *P. aeruginosa* pulmonary infection was realized when a patient with DPB was treated with erythromycin for a prolonged period. This resulted in a dramatic improvement in clinical symptoms, respiratory function and radiographic findings [8]. This astute observation, made by Dr. Shoji Kudoh, lead to a subsequent open trial study which established the clinical effectiveness of long-term erythromycin therapy in DPB patients [9]. Clinical experience in DPB has lead to the use of long-term macrolide therapy in patients with chronic sinusitis, bronchiectasis and CF. While there is mounting evidence of clinical efficacy, the mechanisms of action are still unknown. Currently, investigators are working on two major research directions; 1) macrolide effects on host inflammatory and immune systems, and 2) specific effects of macrolides on the bacteria themselves, including the expression of bacterial virulence factors.

In this chapter, we will review the effects of sub-MIC of macrolides on *P. aeruginosa*, particularly activity of these antibiotics on the bacterial quorum-sensing sys-

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tem; a system that may be crucial in the pathogenesis of chronic *P. aeruginosa* infection. Immunomodulatory properties on host responses and clinical efficacy of macrolides will be more comprehensively addressed in other chapters.

An overview of macrolide antibiotics

The macrolide class of antimicrobials is characterized by a multi-membered lactone ring with one or more amino sugars attached. Macrolides are grouped according to the number of atoms comprising the lactone ring, such as 12-, 14-, 15- and 16-membered rings. The 14-membered ring group includes erythromycin, clarithromycin, roxithromycin and oleandomycin, whereas the 16-membered group contains josamycin, kitasamycin and rokitamycin. The only 15-membered ring is azithromycin, which is characterized by a higher degree of intracellular accumulation within leukocytes and more potent antibacterial activity against gram-negative organisms [10].

Macrolides inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit causing an inhibition of translocation of peptidyl-tRNA and the initial steps of 50S subunit assembly. The spectrum of activity of macrolides includes aerobic gram-positive bacteria, especially *Staphylococcus* spp., and *Streptococcus* spp. A few gram-negative bacteria (e.g., *Campylobacter* spp., *Helicobacter* spp., and *Legionella* spp.), and other atypical pathogens including *Mycoplasma* spp. and *Chlamydia* spp., are also susceptible to this class of antibiotics. In contrast, *P. aeruginosa*, as well as other enteric microorganisms, are intrinsically resistant owing to the exclusion of the macrolide from the cytoplasm by the outer membrane architecture.

Generally, the mode of therapeutic efficacy of antibiotics is attributed to the inhibition of bacterial growth *in vivo* when antibiotic concentrations (usually in serum) exceed the minimum inhibitory concentration (MIC), measured on a short exposure time (generally 24 h) to planktonic forms of the bacteria. However, concentrations below the MIC can still attenuate growth and the expression of a variety of bacterial virulence factors, compromising the ability of the pathogen to cause disease. This activity of antibiotics is referred to as sub-MIC effects. The MIC of macrolides for most *P. aeruginosa* strains is in the range of 128–512 μg/ml (our laboratory data). Peak serum concentrations of erythromycin after a 250 mg oral dose are, however, only 1.0–1.5 μg/ml and the mean sputum concentration after an intravenous dose of 1 g every 12 h was 2–3 μg/ml [11, 12]. Thus, judged by conventional criteria, *P. aeruginosa* is fully resistant to macrolide antibiotics. However, there is increasing evidence of a role of sub-MIC macrolides in suppressing virulence factors of this organism.

A characteristic of macrolides that augment their efficacy is that they can concentrate within leukocytes and can enhance the function of aspects of the cellular immune system [13, 14]. For example, intracellular macrolides may be transported

to the site of an infection, where they are partially released [15]. These data may explain, in part, how relatively higher concentrations of macrolides can occur at the site of infection, as compared to lower levels observed in serum. Furthermore, macrolide accumulation has been demonstrated to occur not only in host cells, but also within bacteria, especially after a prolonged incubation period [16], which may account for sub-MIC effects on pathogens and perhaps clinical efficacy. These data suggests that macrolide antibiotics have the potential for antibacterial activity, not only through direct bactericidal and bacteriostatic effects, but also through suppression of virulence factors.

Macrolide effects on bacteria

The cellular and molecular mechanisms accounting for the dramatic effect of macrolides in DPB patients has been the subject of intensive research. To summarize a large body of work, the clinical efficacy of macrolides in DPB and CF patients is likely attributable to modulation of host inflammatory and immunological pathways and modulation of bacterial virulence factors, such as suppression of exoproducts (e.g., toxins, pigments, alginate) and bacterial cell components (e.g., flagella, pili, lipopolysaccharide [LPS]). In the discussion to follow, we focus on macrolides effects on bacteria, especially sub-MIC macrolide effects on virulence factors of *P. aeruginosa* and its "quorum-sensing" regulatory system.

Sub-MIC effect of macrolides on bacteria and its virulence factors

Suppression of bacterial exoproducts

P. aeruginosa produces a variety of extracellular products, such as pigment, toxins and exopolysaccharide, which contribute to the pathogenesis through cell/tissue destruction, inflammation and other local and systemic effects [17]. Molinari and associates demonstrated that erythromycin, clarithromycin, and azithromycin differed in their ability to inhibit various *P. aeruginosa* virulence factors. Specifically, azithromycin reduced the synthesis of elastase, protease, lecithinase, and DNase to a greater degree than the other macrolides tested, and was the only agent to suppress pyocyanin production [18, 19]. Sato et al. have reported that erythromycin suppresses the production of pyocyanin dose-dependently *in vitro* [20]. Kita and collaborators have reported that erythromycin over a concentration range of 0.1–10 μg/ml suppressed production of elastase, protease and leucocidin in *P. aeruginosa*; although growth of bacteria was not affected significantly during 24 h culture [21]. Sakata and associates have reported that elastase production was inhibited completely by erythromycin in 27 (79.4%) of 34 strains at concentrations between 0.125 and 64 μg/ml [22]. Likewise, Hirakata and colleagues reported that ery-

thromycin suppressed the *in vitro* production of exotoxin A, total protease, elastase, and phospholipase C by *P. aeruginosa* D4 in a dose-dependent manner [23]. A similar investigation confirmed the greater sub-MIC inhibitory activity of azithromycin, as compared to erythromycin, roxithromycin, and rokitamycin against *P. aeruginosa* exoenzymes and exotoxin A [24].

Strains of *P. aeruginosa* involved in chronic lung infection in DPB and CF develop a mucoid phenotype which is attributable to hyperproduction of alginate. These strains transform into a biofilm coating airway surfaces [25]. Within biofilms, bacteria are protected from antibiotics and the host immune system. Sub-MIC of macrolides have been shown to inhibit both the production of alginate and the formation and stability of biofilms [26–28].

Kobayashi has reported that 14- and 15-membered macrolides specifically inhibited the enzyme guanosine diphosphomannose dehydrogenase (GMD), which is involved in the biosynthesis of alginate, but that the 16-membered macrolide midecamycin was ineffective [29]. It is also notable that macrolides can inhibit α-dornase (recombinant human DNase I) with azithromycin displaying greater activity than erythromycin [30].

Several explanations have been proposed for the sub-MIC effects of macrolides on the expression of *P. aeruginosa* exoproducts. This effect may be due to direct inhibition of translation at the ribosomal level, although it is difficult to imagine how the inhibition of enzymes to as low as 30% of normal function would not substantially impact bacterial growth. It has also been suggested that short peptide chains are preferentially more susceptible to macrolides and this would allow for differential inhibition of enzymes [31]. Regardless of mechanisms involved, it does appear that certain macrolides, but not all family members, are active in suppressing virulence factors of *P. aeruginosa*, and this effect is closely linked with those macrolides that demonstrate clinical efficacy, including erythromycin, clarithromycin, roxithromycin and azithromycin.

Bacterial cell surface components and adherence to host cells

The bacterial cell surface components of LPS and outer membrane proteins of *P. aeruginosa* were disrupted when bacteria were grown at sub-MIC of erythromycin or clarithromycin, but not kitasamycin, josamycin, rokitamycin or oleandomycin [32] (Figure 2).

Erythromycin treatment induced reduction of LPS amounts, as determined by the amount of 2-keto-3-deoxyoctulosonic acid, which is a conserved portion of the LPS molecule. Additionally, a reduction of amount of a 38 kDa protein and a concomitant increase of a 41 kDa protein, which are considered to be *Pseudomonas* outer membrane proteins, were demonstrated. Sub-MIC of erythromycin and clarithromycin also rendered *P. aeruginosa* more susceptible to serum bactericidal activity [33]. These alterations of cell surface structures, such as LPS and outer mem-

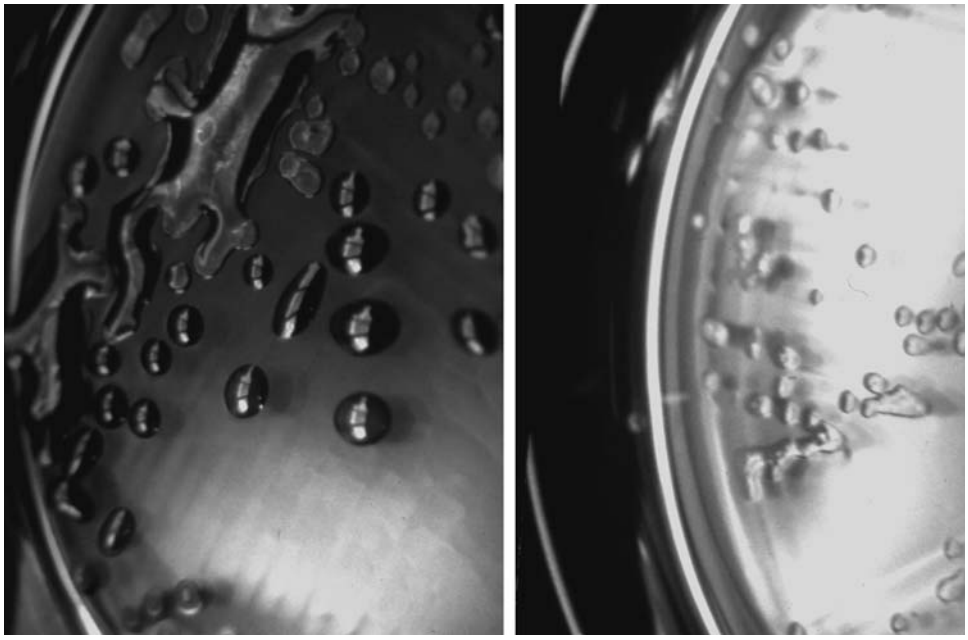
a  b

Figure 1
Colony of mucoid-type P. aeruginosa *grown in agar with (b) or without (a) sub-MIC of erythromycin (10 μg/ml). Smooth colony has changed to rough in the presence of erythromycin, that suggests suppression of exopolysaccharide alginate.*

brane proteins, may facilitate the access of complement to the outer surface, thus increasing bacterial susceptibility.

Tissue invasion requires the attachment of the microorganism to the host cell. Depending on the host site, the microbe will encounter mucosal or epithelial cells to which it must adhere or be eliminated. Gram-negative bacteria attach primarily by means of proteinaceous appendages known as fimbriae and pili, which extend through the mucus layer to bind to the appropriate host receptor. A number of antibiotics have been shown to impair bacterial adherence [34]. Yamasaki and collaborators have provided compelling evidence that exposure of *P. aeruginosa* to erythromycin at 1/4 MIC for only 4 h significantly reduced the number of pili and hence adherence [35]. Another important cell surface structure is flagella, which facilitates bacterial motility and adherence, and enables bacteria to establish a colony in a more hospitable environment. Molinari and associates have reported that erythromycin, clarithromycin and azithromycin inhibited *P. aeruginosa* motility at sub-MIC [18, 19]. Moreover, Kawamura-Sato and collaborators have report-

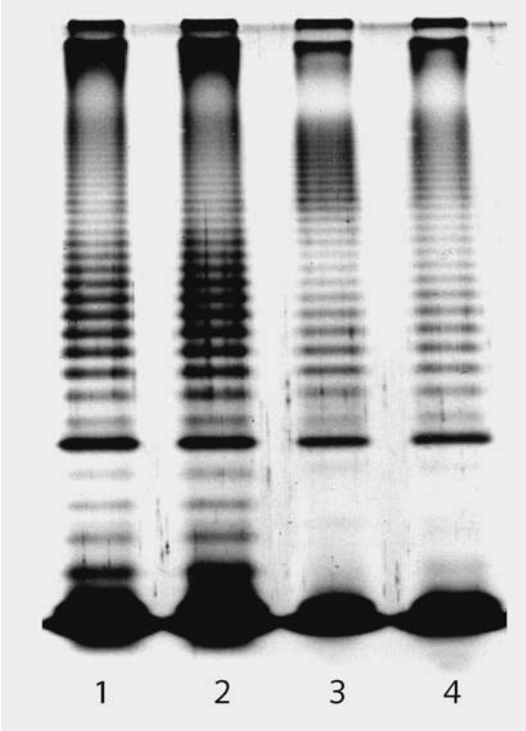


Figure 2
Changes of LPS of P. aeruginosa *grown in agar with sub-MICs of macrolide antibiotics. Lane 1: no antibiotic. Lane 2: josamycin 16 μg/ml. Lane 3: erythromycin 16 μg/ml. Lane 4: azithromycin 4 μg/ml. Change of LPS pattern, especially reduction of lower molecular weight LPS bands, was observed in bacteria grown in the presence of sub-MICs of erythromycin, azithromycin, but not josamycin [32].*

ed that azithromycin can inhibit flagellin expression more effectively than either erythromycin or clarithromycin at concentrations as low as 1/8 MIC [36]. This activity may disrupt biofilm formation in *P. aeruginosa* through inhibition of flagellin expression even at concentrations below the MIC.

Direct killing effects of macrolides with longer incubation

The macrolides do not exhibit intrinsic activity against *P. aeruginosa* based on conventional antimicrobial testing procedures, although appreciable additive and synergistic activities have been observed when macrolides were paired with other antibiotics [37–39]. However, we have reported reduction of viability of *P. aerugi-*

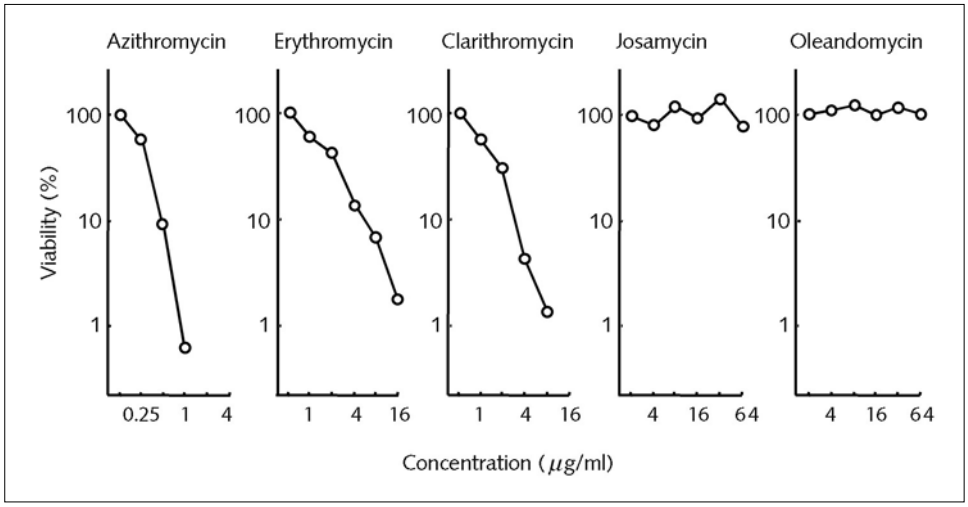


Figure 3
Bactericidal activity of macrolides against P. aeruginosa *after longer incubation*
P. aeruginosa *was incubated on agar with various concentrations of macrolides for 48 hours, and then bacterial viability was compared to that of control bacteria [16].*

nosa when the bacteria were incubated with macrolides for a prolonged time [16]. Exposure to azithromycin for 48 h or more significantly decreased viability of *P. aeruginosa* PAO1 in a concentration-dependent manner, whereas no effect on viability was observed with 24 h or less of incubation. As shown in Figure 3, this time-dependent bactericidal activity was observed with erythromycin, clarithromycin, and azithromycin, but not with josamycin, oleandomycin or other classes of antibiotics (ceftazidime, tobramycin, minocycline, ofloxacin). This reduction in organism viability correlated with a decline in bacterial protein synthesis, which was associated with time-dependent intracellular accumulation of the antibiotic (Fig. 4). Moreover, it is likely that the macrolides may sensitize bacteria to stresses, as these antibiotics induced alterations in a major stress protein, Gro-EL, in both resting and inducible states [40]. These data suggest that conventional antimicrobial susceptibility testing, which is done against planktonic organisms, may not reflect antimicrobial effects of macrolides on *P. aeruginosa* at the site of infection, which may account for discrepancies between clinical efficacy and MIC values.

Figure 5 shows a schematic representing potential effect of macrolides on *P. aeruginosa*. In the respiratory tract or alveolar spaces of patients with persistent *P. aeruginosa* infections, bacteria live on the surface of respiratory cells, where they exist within secreted mucus and host-cell debris in the form of microcolonies or biofilm [41, 42]. As the bacterium multiply, they express virulence factors that may

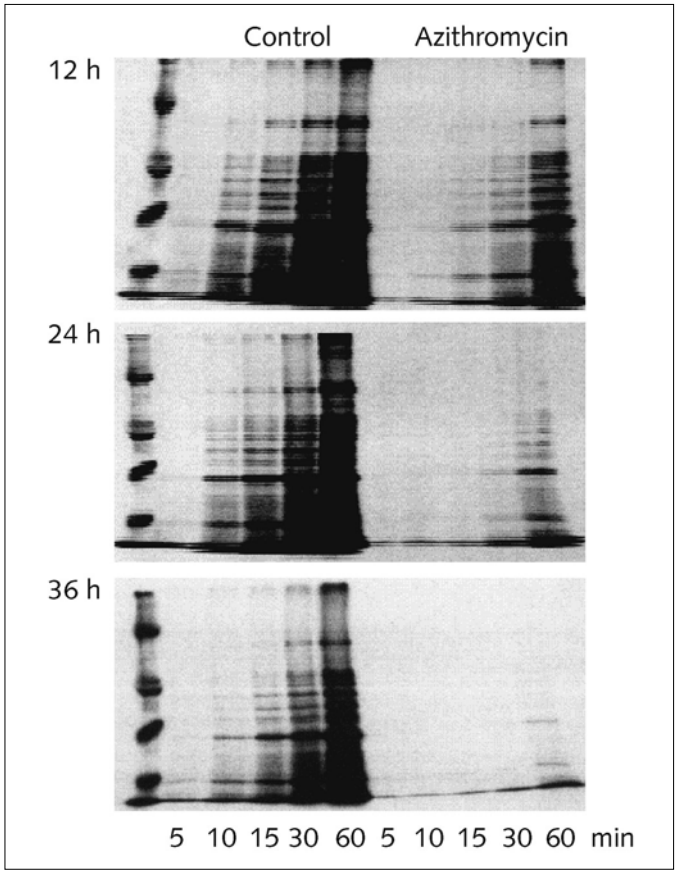


Figure 4
Effects of sub-MIC of azithromycin on protein synthesis of P. aeruginosa
Bacteria was grown on agar with or without azithromycin (4 μg/ml) for 12, 24 or 36 h, and then protein synthesis was examined in a pulse-chase method using ^{3}5S-methionine. Significant suppression of protein synthesis was observed in the presence of azithromycin in a time-dependent manner [16].

injure host cells and induce local host responses, including the production of inflammatory mediators, increases in vascular permeability, and leukocyte accumulation. Bacterial populations directly adhering to epithelial cells may be exposed to high macrolide concentrations due to the generation of antibiotic concentration gradients. Under these conditions, sub-MICs of the drug may suppress the virulence of *P. aeruginosa*. Moreover, in patients undergoing macrolide therapy for prolonged periods, bacteria continuously exposed to the antibiotic may be sensitized to the

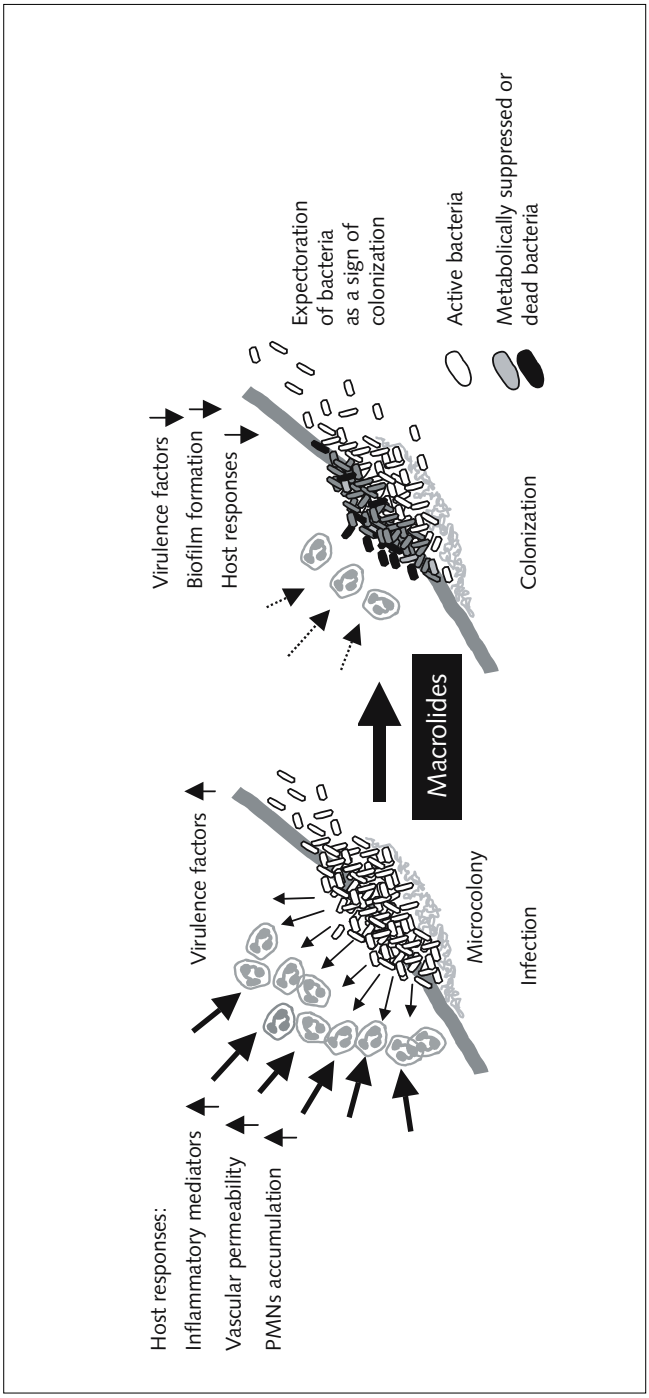


Figure 5: Possible mechanisms of macrolide effects on bacteria

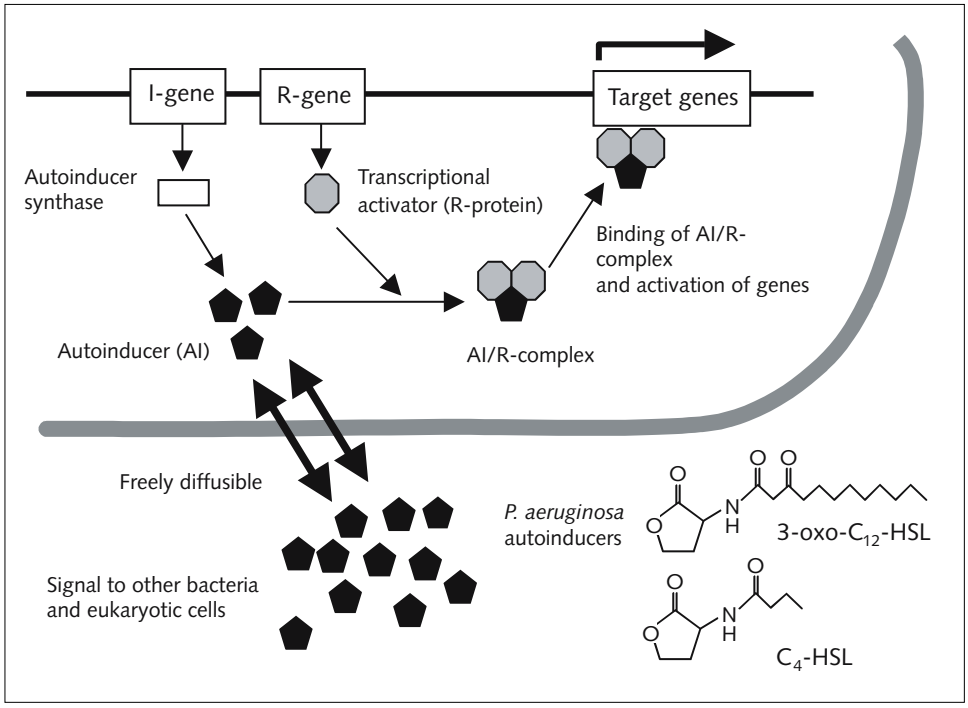


Figure 6
HSL-mediated quorum-sensing systems in bacteria

serum bactericidal effect. Bacteria closely associated with host cells may gradually lose their viability as a consequence of the direct anti-pseudomonal bactericidal activities of these medications. In addition, macrolides may disrupt biofilm attachment to host epithelium. Thus, we speculate that long-term macrolide therapy may shift the host-pathogen interaction from infection to a relatively benign colonization state and possibly even to eradication in some patients. This hypothesis is consistent with the common clinical observation that long-term macrolide therapy leads to improvements in clinical symptoms and laboratory data before any observable bacteriological response.

Quorum-sensing systems as new therapeutic targets

Role of quorum-sensing systems in chronic pulmonary P. aeruginosa infection
P. aeruginosa possesses at least two separate but interrelated quorum-sensing systems, *las* and *rhl* [43, 44]. As the bacterial population increases, the autoinducer

signal molecules, 3-oxo-C_{12}-homoserine lactone (HSL) and C_4-HSL, accumulate in the environment. When the concentration of autoinducer reached to a threshold in bacteria, these molecules bind to and activate their cognate transcriptional regulators (Fig. 6). Both systems have been found to regulate multiple virulence factors, such as extracellular toxins (e.g., elastase, alkaline protease, exotoxin A), rhamnolipid and pyocyanin. To investigate the effects of quorum-sensing systems during infections, strains of *P. aeruginosa* that contain deletions in one or more of the quorum-sensing genes were tested in various infection models, including a burn injury mouse model, a murine model of acute pneumonia and a rat model of chronic lung infection [45–48]. A general observation obtained from these models indicates that strains containing a mutation in quorum-sensing genes were less virulent as compared with wild-type *P. aeruginosa*. Another interesting aspect in quorum-sensing research is the contribution and association of this system in biofilm formation. Accumulating data demonstrated that quorum-sensing systems are essential for differentiation and maturation within biofilm in *P. aeruginosa* infection [49–53].

Quorum-sensing is functionally active during *P. aeruginosa* infections in humans. Sputum samples obtained from CF patients chronically infected with *P. aeruginosa* contain mRNA transcripts for the quorum-sensing genes [54]. Sputum from *P. aeruginosa*-infected CF patients also contains the autoinducer molecules 3-oxo-C_{12}-HSL and C_4-HSL [49]. These autoinducer molecules were directly extracted and measured in CF sputum [55]. These samples contained approximately 20 nM 3-oxo-C_{12}-HSL and 5 nM C_4-HSL. In contrast, when bacteria were grown in a biofilm, considerably higher concentrations (300–600 μM) of 3-oxo-C_{12}-HSL were measured [56]. Although it is difficult to define exact concentrations of autoinducer molecules at the site of infection, particularly in biofilm, these results demonstrate that quorum-sensing systems may be active during *P. aeruginosa* infection and potentially regulate the expression of various genes *in vivo*.

Accumulating evidence suggests that the quorum sensing signal molecule 3-oxo-C_{12}-HSL is also a potent stimulator of multiple eukaryotic cells and thus may modulate the host inflammatory response during *P. aeruginosa* infection. *In vitro* experiments have shown that 3-oxo-C_{12}-HSL stimulates the production of the inflammatory chemokine IL-8 from human lung bronchial epithelial cells [57, 58]. In addition, Smith et al. have reported that 3-oxo-C_{12}-HSL could stimulate a complex response *in vivo* by inducing several inflammatory cytokines and chemokines [47]. More recently, we have reported that 3-oxo-C_{12}-HSL from a concentration of 12 μM induces apoptosis in certain types of cells, such as macrophages and neutrophils, but not in epithelial cells [59] (Fig. 7). Taken together, these data suggest that the quorum-sensing molecules have a critical role in the pathogenesis of *P. aeruginosa* infection, not only in the induction of bacterial virulence factors but also in the modulation of host responses. The role of bacterial quorum-sensing systems and their regulation in infection have been reviewed elsewhere [60–63].

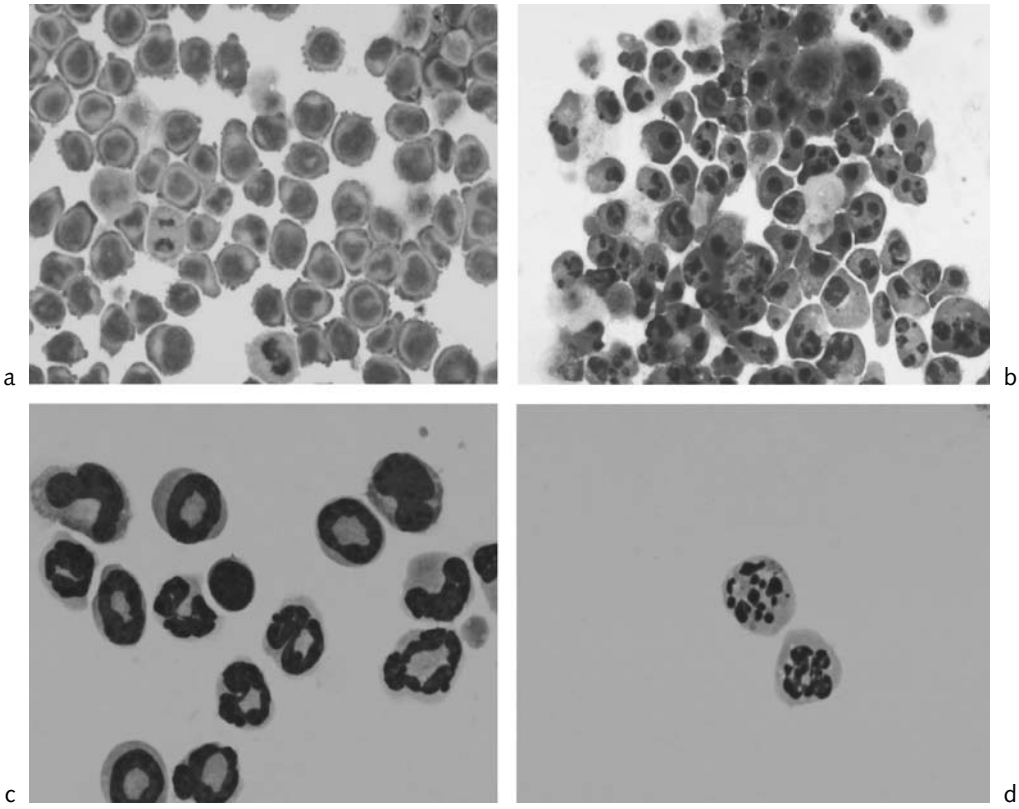


Figure 7
Induction of apoptosis by Pseudomonas *3-oxo-C_{12}-HSL in macrophage and neutrophil*
Macrophage cell line U-937 and mouse neutrophil were incubated with or without 3-oxo-C_{12}-HSL, and then morphology of cells was examined at 4 h after incubation.
a: U-937 cell, control. b: U-937 cell, 3-oxo-C_{12}-HSL. c: neutrophil, control. d: neutrophil, 3-oxo-C_{12}-HSL [59].

Potential of macrolides as quorum-sensing inhibitors

The discovery that gram-negative bacteria employ HSL autoinducer molecules to globally regulate the production of virulence determinants has identified a novel target for therapeutic intervention. The ability to interfere with bacterial virulence by jamming signal generation or signal transduction is intellectually seductive and pharmaceutically appealing, and may also be of considerable clinical importance. Strategies to inhibit quorum-sensing systems include chemical antagonists and specific antibody to inhibit the autoinducers, HSL-destroying enzyme lactonase, and

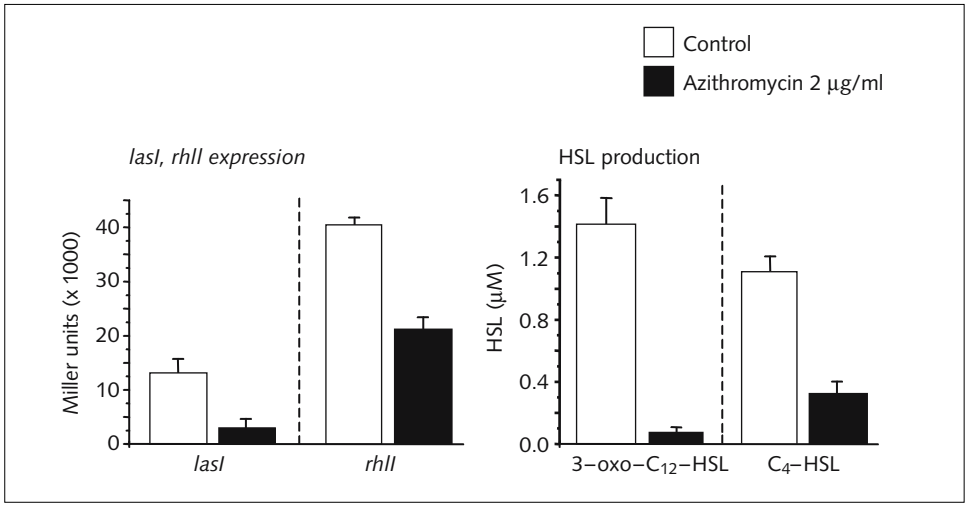


Figure 8
Effects of azithromycin on quorum-sensing systems of P. aeruginosa
P. aeruginosa *was incubated with or without azithromycin 2 μg/ml for 10 hours, and then autoinducer synthase expression (*lasI, rhlI*) and HSL production were examined [67].*

suppression of quorum-sensing by interfering with associated genes and gene products. Several investigators have reported the feasibility of HSL-analogues [64, 65] and synthetic derivatives of natural furanone as means to inhibit bacterial quorum-sensing systems [66].

Clinical and experimental data described above provided a hint that certain macrolides and their analogues may function as *Pseudomonas* quorum-sensing inhibitors. As shown in Figure 8, 2 μg/ml of azithromycin significantly suppressed transcription of *lasI* by 80% and *rhlI* by 50% in *P. aeruginosa* PAO1 [67]. Additionally, the production of 3-oxo-C_{12}-HSL and C_4-HSL was inhibited to approximately 6% and 28% of the control, respectively. In contrast, azithromycin treatment did not alter the expression of the *xcpR* gene, which codes for a structural protein belonging to the type II secretion pathway. These data suggested that azithromycin suppressed quorum-sensing systems in *P. aeruginosa*, and azithromycin's effects on these bacteria are somewhat selective in nature. Importantly, we have observed suppression of *lasI* gene expression by erythromycin, clarithromycin and roxithromycin, but not by oleandomycin and josamycin. These results suggested that the clinically effective macrolides are also the macrolides that are active in suppressing quorum-sensing system, and are consistent with the notion that macrolides might reduce the production of *Pseudomonas* virulence factors by inhibiting the synthesis of the autoinducer molecules.

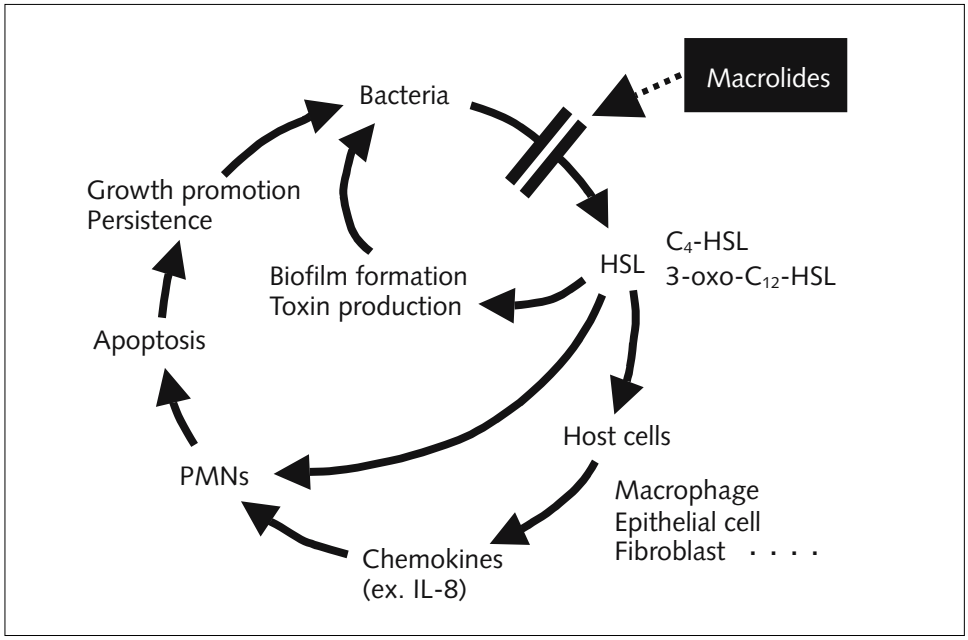


Figure 9
Inhibition of HSL production by macrolides and its impact on pathogenesis of chronic P. aeruginosa *pulmonary infection [59].*

Figure 9 demonstrates several potential mechanisms by which macrolide antibiotics may suppress quorum-sensing systems and highlight their contribution to clinical efficacy in chronic *P. aeruginosa* pulmonary infections. Activation of the quorum-sensing cascade promotes biofilm formation at the site of infection, which make conditions more favorable for bacterial persistence in the lung. Bacterial autoinducers, especially 3-oxo-C_{12}-HSL, stimulates several types of cells, such as epithelial cells, fibroblasts, and macrophages, to induce production of neutrophil chemotactic factors (IL-8 in humans and MIP-2 in mice). Migrated neutrophils are triggered to produce several toxic substances for killing of bacteria, but these molecules, in conjunction with bacterial virulence factors, promote tissue destruction that is a hallmark of the lungs of CF patients. In sites where bacteria are actively producing autoinducers and autoinducer-regulated virulence factors, host cells come in contact with these bacterial factors. In these sites, neutrophils begin to undergo apoptosis, and this process may be accelerated by the presence of bacterial factors, such as 3-oxo-C_{12}-HSL. Apoptotic neutrophils, in addition to secreted mucus and other cell debris, may serve as nutrients for the growth of bacteria and a niche for