NOVEL CELLULAR LUMINESCENCE PROBES FOR IMMUNOLOGICAL AND TOXICOLOGICAL ASSESSMENTS

by

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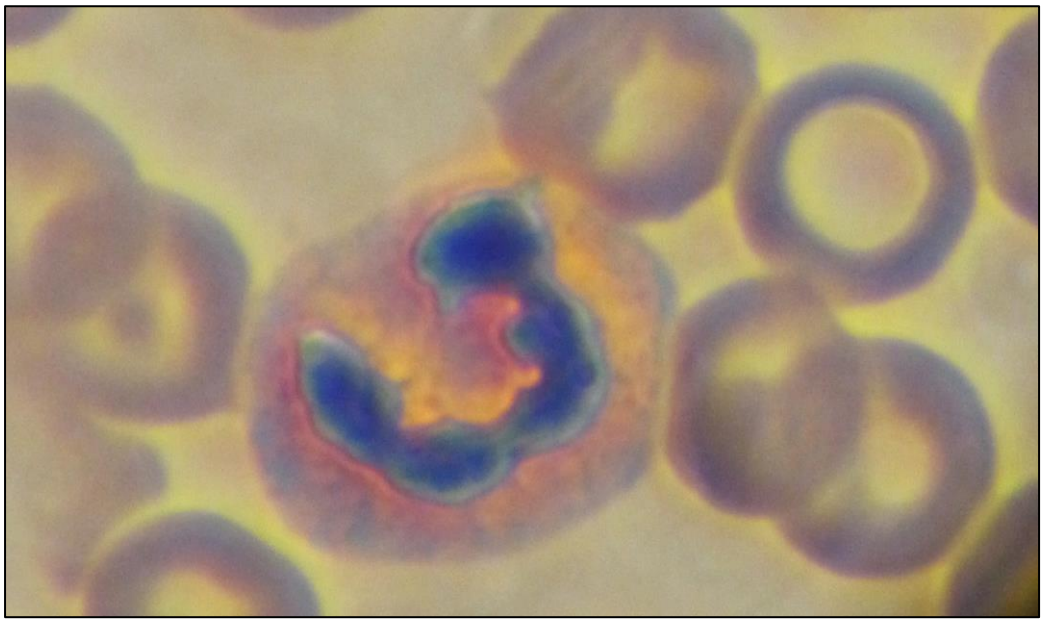

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To my parents and all the others so dear to me!

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ABSTRACT

Escherichia coli K-12 (*pEGFPluxABCDEAmp*) (*E. coli*-lux), constitutively emitting bioluminescence (BL), was constructed and its BL emitting properties tested in different growth and killing conditions. The BL emission directly correlated with the number of viable *E. coli*-lux cells, and when subjected to the antimicrobial agent, the diminishment of the BL signal was linked directly to the number of killed bacterial cells. The method provided a very convenient application, especially when compared to conventional plate counting assays. This novel real-time based method was utilized in both immunological and toxicological assessments.

The parameters such as the activation phase, the lytic phase and the capacity of the killing of the serum complement system were specified not only in humans but also in other species. *E. coli*-lux was also successfully used to study the antimicrobial activities of insect haemolymph.

The mechanisms of neutrophil activity, like that of a myeloperoxidase (MPO)-H_2O_2-halide system, were studied using the *E. coli*-lux approach. The fundamental role of MPO was challenged, since during the actual killing in described circumstances in phagolysosome the MPO system was inactivated and chlorination halted.

The toxicological test system, assessing indoor air total toxicity, particularly suitable for suspected mold damages, was designed based on the *E. coli*-lux method. Susceptibility to the vast number of various toxins, both pure chemicals and dust samples from the buildings and extracts from molds, were investigated. The *E. coli*-lux application was found to possess high sensitivity and specificity attributes. Alongside the analysis system, the sampling kit for indoor dust was engineered based on the swipe stick and the container. The combination of practical specimen collector and convenient analysis system provided accurate toxic data from the dust sample within hours.

Neutrophils are good indicators of the pathophysiological state of the individual, and they can be utilized as a toxicological probe due to their ability to emit chemiluminescence (CL). Neutrophils can either be used as probe cells, directly exposed to the agent studied, or they can act as indicators of the whole biological system exposed to the agent.

Human neutrophils were exposed to the same toxins as tested with the *E. coli*-lux system and measured as luminol amplified CL emission. The influence of the toxins on the individuals was investigated by exposing rats with moniliniformin, the mycotoxin commonly present in Finnish grains. The activity of the rat neutrophils was found to decrease significantly during the 28 days of exposure.

ABSTRAKTI

Escherichia coli K-12 transformoitiin uudella bakteerilusiferaasigeenikasetin sisältämällä *pEGFPluxABCDEAmp* plasmidilla. Aktiivinen bakteerilusiferaasioperoni tuottaa konstitutiivista bioluminesenssia emittoivia *E. coli* soluja, ilman lisättyä substraattia. Uusi konstruktio nimettiin *E. coli*-lux:si ja sen bioluminesenssiemissio-ominaisuuksia mitattiin erilaisissa inkubaatio-olosuhteissa. Tutkimuksissa havaittiin *E. coli*-lux:in tuottaman bioluminesenssin olevan suoraan verrannollinen reaktioseoksessa olevien elävien bakteerisolujen lukumäärään ja kun reaktioon lisättiin antimikrobiaalista yhdistettä, valosignaalin laskun havaittiin korreloivan kuolleiden bakteerisolujen lukumäärän kanssa. Konventionaaliseen maljalaskentamenetelmään verrattuna *E. coli*-lux – sovellus tarjosi erittäin käytännöllisen vaihtoehdon bakteerien elinkyvyn ja tapon mittaamiseen ja sitä hyödynnettiin tässä kirjassa esitellyissä immunologisissa ja toksikologisissa tutkimuksissa koetinorganismina.

Synnynnäisen immuniteetin tutkimuksessa *E. coli*-lux konstruktiota hyödynnettiin seerumin komplementtisysteemin aktivaatioreaktioteiden kineettisten parametrien kuten aktivaatio- ja lyyttisen faasin nopeuden sekä bakteeritappokapasiteetin määrittämiseen ihmisen, kirjolohen, sian, rotan, sekä lepakon seerumeista. Komplementin komponenteille spesifisten monoklonaalisten vasta-aineiden sekä antiseerumeiden avulla selvitettiin tiettyjen komponenttien vaikutusta tapporeaktioon. Lisäksi *E. coli*-lux menetelmää käytettiin hyönteisten veren, hemolymfan, antimikrobiaalisten ominaisuuksien selvittämisessä.
Polymorfonukleaaristen leukosyyttien, eli neutrofiilien aktiivisuutta, fagosytoosia ja myeloperoksidaasi (MPO) -vetyperoksidi (H_2O_2) -kloridi-systeemin roolia fagolysosomaalisessa mikrobitapossa tutkittiin *in vivo* ja *in vitro* olosuhteissa, käyttämällä koettimena *E. coli*-lux soluja. MPO-systeemin perinteinen rooli neutrofiilien pääasiallisena bakteeritappomekanismina kyseenalaistettiin, koska olosuhteissa jossa mikrobitappo tapahtuu, MPO:n klorinaatioaktiivisuus havaittiin olemattomaksi. Tulokset viittasivat H_2O_2:n fagolysosomaalisen konsentraation itsessään olevan riittävän suuri mikrobitappoon.
Neutrofiilit toimivat myös hyvinä nisäkäsyksilön patofysiologisen tilan indikaattoreina ja niitä voidaan hyödyntää koetinsoluina toksikologisessa tutkimuksessa. Neutrofiilien kyky emittoida kemiluminesenssiä (CL) fagosytoosin hengitysryöpsähdyksen yhteydessä mahdollistaa solujen toiminnan havainnoinnin levynlukijalumienometrillä. Toksinen vaikutus havaitaan CL signaalin laskuna. Ihmisen neutrofiilejä altistettiin suoraan toksiineille ja niitä käytettiin *E. coli*-lux-systeemin toksisuusmittauksissa referenssikoettimina. Neutrofiilit voivat myös toimia koko altistetun yksilön systeemin indikaattoreina ja niitä käytettiin tutkittaessa suomalaisista viljoista eristetyn mykotoksiinin, moniliformiinin, vaikutusta rottien immuunipuolustukseen 28 päivää kestäneessä subkliinisessä kokeessa. Oraaliasuspensiona annosteltu moniliformiini laski merkitsevästi rottien neutrofiilien aktiivisuutta.

E. coli-lux konstruktiota käytettiin perustana uuden, rakennuksen sisäilman kokonaistoksisuutta mittaavan menetelmän kehitykseen. Testi perustuu kohteista kerättyjen sisäpölynäytteiden analysointiin, jossa *E. coli*-lux altistetaan pölyistä tehdyille H_2O- ja DMSO- uutoksille ja toksisuus havaitaan valoemission heikkenemisenä. Toksisuus ilmoitetaan EC_{50} arvoina mg pölyä/ml uutosnestettä kohden. Menetelmä pystyy tunnistamaan kosteus- ja homevaurioiden sekä muiden sisäilmaongelmia aiheuttavien myrkkylähteiden toksisuuden. Analyysimenetelmän spesifisyys ja sensitiivisyys on verrannollinen perinteisiin mammaliansoluilla tehtäviin toksisuustesteihin ja lisäksi *E. coli*-lux systeemi on edullisempi ja nopeampi. *E. coli*-lux menetelmällä löydetty toksisuus korreloi tilankäyttäjien terveyshaittojen kanssa.
Analyysimenetelmän ohella on kehitetty uusi näytteenottomenetelmä, jossa huonepölyä kerätään (1 – 10 mg) kuitupuikolla pyyhkäisemällä, useasta eri kohdasta. Kukin yksilöity pölynäyte suljetaan

mukana olevaan putkeen ja toimitetaan laboratorioon, jossa *E. coli*-lux preparaatti altistetaan usealle eri näytelaimennokselle. Toksisuus ilmoitetaan EC_{50} arvoina mg pölyä/ml uutosnestettä kohden ja/tai prosentuaalisena vertailuarvona tunnetun puhtaan toksiinin aiheuttamaan myrkyllisyyteen.

ABBREVIATIONS

AMP	ampicillin
AP	alternative pathway
APC	antigen presenting cell
ATP	adenosine triphosphate
BL	bioluminescence
CD	cluster of definition
CFU	colony forming unit
CHP	chloramphenicol
CL	chemiluminescence
CP	classical pathway
CPS	counts per second
CRP	C-reactive protein
DMSO	dimethyl sulfoxide
DON	deoxynivalenol
EC	effective concentration
EDTA	ethylenediamine-tetraacetic acid
EGFP	enhanced green fluerescence protein
EGTA	ethyleneglycol-tetraacetic acid
FMNH	flavin mononucleotide
GFP	green fluorescencent protein
Gram (+/-)	gram staining differentiates bacteria by the properties of the cell wall
HBSS	hanks balanced salt solution
LB	luria broth
LD	lethal dosage
LP	lectin pathway
LPS	lipopolysaccharide
lux	bacteria luciferase gene
M9	M9 minimal media
MAC	membrane attack complex
MASP	mannan binding lectine serine peptidase
MBL	mannose binding lectin
MIC	minimal inhibitory concentration
MLC	minimal lethal concentration
MON	moniliformin
MPO	myeloperoxiadase
NADPH	nicotinamide adenine dinucleotide phosphate
NOD	nucleotide-binding oligomerization domain
OD	optical density
PAMP	pathogen associated molecular patterns
PHMG	polyhexamethylene biguanide
PmB	polymyxin B
PMN	polymorphonuclear neutrophil
SOD	superoxide dismutase
ROS	reactive oxygen species
TA	toxin-antitoxin-system
TLR	toll like receptor
VBNC	viable but not culturable

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications and manuscripts, referred to in the text with Roman numerals.

I Kilpi, M.K., Atosuo, J.T. & Lilius, E.M. 2009, ”Bacteriolytic activity of the alternative pathway of complement differs kinetically from the classical pathway“, *Dev Comp Immunol*, vol. 33, no. 10, pp. 1102-10.

II Atosuo, J., Lehtinen, J., Vojtek, L. & Lilius, E.M. 2013, ”Escherichia coli K-12 (pEGFPluxABCDEamp): a tool for analysis of bacterial killing by antibacterial agents and human complement activities on a real-time basis“, *Luminescence*, 28, 5, 771-779

III Lilley, T.M., Ruokolainen, L., Meierjohann, A., Kanerva, M., Stauffer, J., Laine, V.N., Atosuo, J., Lilius, E-M., Nikinmaa, M. 2013 ”Resistance to oxidative damage but not immunosuppression by organic tin compounds in natural populations of Daubenton's bats (Myotis daubentonii)” *Comp.Biochem.Physiol.C.Toxicol.Pharmacol*., 157, 3, 298-305

IV Vojtek, L., Dobes, P., Buyukguzel, E., Atosuo, J. & Hyrsl, P. 2014, ”Bioluminescent assay for evaluating antimicrobial activity in insect haemolymph“, *Eur J. Entomol*, vol. 111(3), pp. 335-340.

V Atosuo, J.T., Lilius, E.M. 2011 ”The real-time-based assessment of the microbial killing by the antimicrobial compounds of neutrophils“, *The Scientific World Journal*, vol. 11, pp. 2382-2390.

VI Lilius, E-M., Atosuo, J. 2015 ”Myeloperoxidase in phagolysosome is not needed for killing ingested bacteria“ *Manuscript*

VII Jonsson, M., Atosuo, J., Jestoi, M., Nathanail, A.V., Kokkonen, U-M., Anttila, M., Koivisto, P., Lilius, E-M., Peltonen, K. 2015 ”Repeated dose 28-day oral toxicity study of moniliformin in rats“ , Feb 17;233(1):38-44.

VIII Mikkola, R., Andersson, M., A., Atosuo, J., Kredics, L., Marik, T., Lilius, E-M., Markkanen, P., Järvi, K., Hirvonen, M-R., Huttunen, K., Alenius, H., Matikainen, S., Leino, M., Hautaniemi, M., Salo, S., Salin, P., Salkinoja-Salonen, M. 2015 **”** Tracking mycotoxin producing indoor molds “ *Manuscript*

A selection of unpublished data is presented in the results and discussion section.

1. INTRODUCTION

Our aim was to analyze the antimicrobial effects of the innate immune mechanisms like serum complement system and especially the phagocyte activity of neutrophils. Like almost everything in the field of microbiology, these studies were previously operated with the plate counting method lacking in kinetics and real-time features. The studies described in this book started in 2008 by assaying the viability and the bioluminescence emission of a new *Escherichia coli* K12 (*E. coli*-lux) construct. This novel approach included the neglected real-time features and provided a new and practical approach for the assessment of bacterial viability and antimicrobial reactions. Numerous antimicrobial agents and their killing features were tested, studied and results recorded in order to clarify different antimicrobial mechanisms. After this groundwork, the gained data was utilized for the assessment of the antimicrobial features of human serum. It is noteworthy that not only the human complement pathways were assayed but also those of fish, mice, rats, pigs, bats, some birds and moreover, the antimicrobial properties of the haemolymph from various insects.

The other field of our professional interest was and is phagocytosis and especially neutrophil activity. Like in the many fields of science, also in immunology, seemingly established ideas, like what actually kills the target inside the neutrophil phagolysosome, becomes obsolete and in need of revision. A bacterial cell, the natural target for phagocytes, with suitable reporter genes came in handy, and *E. coli*-lux was used to study the phagolysosomal killing activity with neutrophils *in vivo* and also with cell-free myeloperoxidase (MPO)-hydrogen peroxide-halide system *in vitro*. During the period of 2009–2014, we focused on the role of the oxidative killing reaction in neutrophils by showing that the MPO is obviously not in as fundamental role in a bacterial killing in the neutrophil phagolysosome as has been previously dictated.

Meanwhile, we participated in a Finnish Academy project with the Finnish Food Safety Authority EVIRA, discovering the physiological effects of the toxins from molds discovered in Finnish grains. Our task in this coalition was to reveal the effects of mycotoxins on neutrophil activity. This project culminated in an extensive one-month experiment in the spring of 2010, when more than 40 exposed rats were sampled and studied. Moniliformin (mycotoxin) was found to decrease the rats' neutrophil phagocytic activity.

During and after the antimicrobial experiments with different toxins, antibiotics, solvents and immunological systems, we realized that the *E. coli*-lux method is very applicable in toxicological assessment. When being asked, it felt natural to participate in the TOXTEST project during 2010–2012 with the National Institute of Healt and Welfare and the Finnish Institute of Occupational Health. The Ministry of Social Affairs and Health funded the project with the aim of finding a reliable application for testing indoor toxicity. The focus was in mold-damaged buildings. During this quarrelsome project, we developed an application for indoor toxicity measurement utilizing *E. coli*-lux as a toxicological probe. This novel *in vitro* assay not only correlated with the level of damage observed in the buildings but also with the health survey results collected from the users of the damaged buildings.

The literature review in this book is divided into three sections: The first part focuses on immunology, mainly the characteristics of complement system and neutrophil activity. The second part is a microbiological section describing the characteristics of bacterial cell existence, growth, viability and death. The third section covers the principals of the measurements and applications used in toxicological assessments.

These subjects form a path of topics I followed during my PhD student years in three interesting projects. While it is too much to hope that the selection of new material avoids criticism, an earnest effort has been made to include the truly significant and fundamental advances.

Janne Atosuo 2014

2. LITERATURE REVIEW

2.1 Innate immune system

Our system encounters a huge number of different pathogenic microbes that only very occasionally cause diseases. These threats are normally recognized and destroyed by the defense mechanism, which does not activate the clonal expansion of the adaptive immune system by antigen specific lymphocytes. This first line of defense is constructed by the innate immune system and, despite the more non-selective manner of target recognition compared to adaptive immune systems, it very efficiently discriminates between the host and pathogenic cells providing an effective initial defense machinery while also participating, as an irreplaceable element, in the functions of adaptive immune responses (1).

Innate systems, such as phagocytizing leukocytes (neutrophils, macrophages, dentritic cells), the soluble parts of blood like the complement system and other extracellular fluids (antimicrobial enzymes, lysozyme, antimicrobial peptides) are the main tools for destroying bacterial and fungal pathogens. The role of the adaptive system is to augment this procedure by producing enhanced specificity and memory. The importance of the innate immune system is sometimes understated, but defects in this mechanism are usually lethal, even in the presence of an intact adaptive system (1, 2).

The first step in an initial encounter with a pathogen after it has passed through the host's anatomic barriers is an immediate onset of the soluble mechanisms, either killing or weakening the intruder. Specific effectors perform the recognition. The complement system in the blood both lyses the intruding pathogens and targets them for phagocytosis by opsonization. If the target is destroyed the removal of the infectious agent and the waste material is conducted by phagocytotic cells, like neutrophils (3). If the pathogen can breach this first humoral step of defense, effector cells are recruited. They are activated by target recognition in pathogen associated molecular patterns (PAMP), the structure's presence in the pathogen but absence in the host's structures. Neutrophils have receptors for complement components on the pathogen surface (opsonization). After recognition and activation, the infectious agent is effectively phagocytized and discarded (3).

The third step in this context is the activation of the adaptive arm of the immune system. It has been estimated that more than 99 % of all encounters with pathogens remain subclinical, with no adaptive response or induced immunological memory. In addition to neutrophils, there are also macrophages and dentritic cells capable of phagocytosis. These cells are found in tissues and they act as an antigen-presenting cell (APC) (2, 3).

These cells recognize their targets, activate and phagocytose the invading pathogen. They process antigen structures, migrate from the site of infection to the lymphoid tissue, where they present these particles to the T and B lymphocytes, and secrete a wide variety of cytokines, inducing the activation of helper and effector T-cells, antibody production from B cells and finally the pool of both T and B memory cells. These antigen-specific lymphocytes also lead to the formation of specific memory cells providing long-lasting specific immunity against this antigen.

2.1.2 Complement system

The complement system is the primary mediator of the immune response against invaders. It consists of more than 30 chemically and immunologically distinct blood (also present in other body fluids) proteins and glycoproteins capable of interacting with each other, with cell membranes and with antibodies. It has a distinct self-nonself-discrimination system in order to prevent cell and tissue damage caused by the auto-activation of the system (1, 2, 4) (I, II, III).

Complement proteins are mainly produced in the liver. The activation of this system leads to a protease cascade, where the previous component always acts as a proteolytic enzyme, cleaving and activating the next component (Figure 1). The cascade is expedited and amplified in every step, since one upstream component can activate multiple downstream components in the cascade.
The activation of the system leads to the generation of biological activity ranging from direct proinflammatory regulation to the formation of the membrane attack complex (MAC), causing the lysis of target cell bacteria, fungi and viruses. In addition, the complement is able to recruit and enlist the participation of other humoral and cellular effector systems. These include the induction of histamine release from mast cells, the regulation of leukocyte migration (chemotaxis) and enhancement phagocytosis by opsonization. Self-nonself-discrimination of the complement system is due to its complex passive and active mechanisms, mainly regulating the protein stability of the components. The process of opsonization is a means of identifying the invading microbes by phagocytes. The nonspecific binding of complement components C3b and C3bi and the specific binding of the serum antibodies to the invading pathogen are normally required for the successful recognition and destruction of this pathogen by the phagocytic cell (5-7).
The individual proteins of the complement system are normally present in the circulation as functionally inactive molecules. The native precursors are designated by numbers, complement component 1 (C1) to C9 or, in the case of some of the components, by symbols or trivial names such as properdin, factor B and D. Each component must be activated sequentially under appropriate conditions in order for the complement reaction to progress.
The complement system also contributes to the adaptive immune system. Target opsonization leads to phagocytosis by APC macrophages and dentritic cells expressing complement receptors. After phagocytosis, these leukocytes travel to the lymphoid tissues, where they present the processed antigen with their major histocombability complex II (MHC II) receptor and enhance the presentation to antigen specific T-cells (8).

B-cells have receptors for the complement component; CR 2 (human complement component receptor type 2) recognizes the complement coated antigen and enhances the B-cell activation against this antigen (9).
There are 3 independent pathways leading to the activation of the terminal, the biologically important portion of the complement sequence (Figure 1). Different substances trigger these mechanisms of activation, termed the classical, alternative and lectin pathways. Each involves several reaction steps.
The classical reaction pathway is activated by antibody-antigen complexes and is thus a part of the adaptive immune response, while lectin and the alternative pathway react to the structures of certain molecules and motifs on the target surface. In the classical pathway, the advantage is in the accuracy of the recognition with the antibodies, although the formation of these molecules can take weeks during the primary response. The alternative pathway is thought to be phylogenetically the oldest activation pathway, formed before the age of the adaptive response**.** When encountering the potential pathogen, the alternative pathway is very rapidly activated. This is because of the "tickover" reaction, the continuous spontaneous activation of the alternative pathway in the minor scale. In the lectin pathway, the recognition takes place with the mannose binding lectin sticking to the mannan or N-acetylglucosamide residues on the microbe cell membrane (2, 4).
All activation pathways converge at the midpoint of the complement system, and the reaction sequence, involving reactions of C5 through C9 and to the formation of the MAC, is common to all three pathways (Figure 1). MAC forms a channel through the targets plasma membrane and alters the permeability of the target cell allowing the influx of the extracellular fluids and phagolysosomal ingredients such as lysozyme and outflow of the amino acids, potassium, ATP, causing lysis or weakening of the target cell (Figure 1) (2).

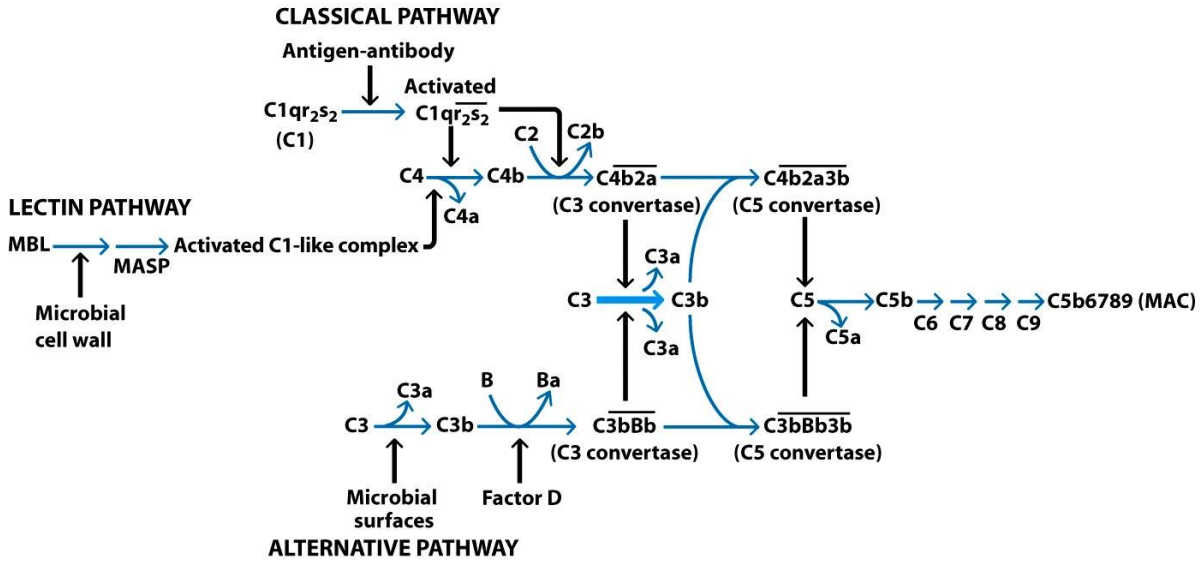

Figure 7-2
Kuby IMMUNOLOGY, Sixth Edition
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Figure 1: The serum complement system. (*Kuby* IMMUNOLOGY, 6. Edition 2007)

Classical pathway

The classical activation pathway of the complement was the first one to be discovered at the end of the 19th century (10). The designation complement refers to the heat labile compartment of serum and its ability to recognize, opsonize and kill the targets non-specifically, thus completing the work of the immune cells (I, II).

The classical pathway (CP) is activated by antigen-antibody complexes or aggregated immunoglobulins connecting this pathway to adaptive immunity. The most powerful activators are immunoglobulin G1 (IgG1), IgG3, IgM and IgG2 respectively, IgG4, IgA, IgD and IgE are weak activators. The C-reactive protein (CRP), bound to the microbe surface, also initiates this classical pathway.

The complement component 1 complex (C1) acts as a recognition molecule (Figure 1). C1 is a complex molecule consisting of one C1q and two serine proteases C1r and C1s, circulating in the blood. The calcium (Ca^{2+}) dependent association of molecules holds this complex together (2, 11).

Allosteric turns in C1q, after the C1-complex has bound to the target antibody, activate the enzyme activity of C1r, which in turn activates C1s enzyme activity. C4 and C2 act as substrates for C1s, and C4 is cleaved into two parts, C4a and in bigger protein C4b, which attaches covalently to the target surface. C2 binds to the surface bound C4b, and C2b is cleaved from this complex. The active protelytic enzyme C4bC2a is the main product of the classical activation pathway capable of cleaving numerous C3 and C5 molecules and thus activating the final lytic pathway. Cleaved C3a and C5a components are powerful inflammation transmitters, anaphylatoxins, and chemotaxins (2).

Formed C3b has a high affinity to bind covalently to the target surface, close to C3 and C5 convertase (C4bC2a), and one of the main functions of C3b is to pluck C5 from the plasma and provide them for C4bC2a (Figure 1). The MAC formation is initiated by the cleavage of C5 into C5a and C5b, the latter acting as a starting point for this lytic complex. C6 and C7 bind to C5 forming a partially hydrophobic complex C5b67 attached to microbe surface. C8 and multiple (3-18) C9 molecules attach to this complex on the surface. Attachment alters the shape of these molecules from

spherical to a more rod-like shape, penetrating through the plasma membrane. MAC is formed (Figure 2) (2, 12)
Immune complexes and chromatin (DNA-histone complexes) can activate the classical pathway. This activation mainly proceeds to the formation of C3b. C3b and C4b molecules attach covalently to these waste complexes and enhance phagocytosis. Defects in C2 and C4 production can thus lead to diseases where immune complexes and other aggregates can accumulate in blood circulation and joints. C1q is also known to bind to exposed phospholipids on the surface of apoptotic cells, participating in the elimination of damaged cells from tissues.
CRP, an acute phase protein, is also known to activate the classical pathway. Activation utilizing CRP is thought to be useful in the primary microbe encountering when the adaptive system has not yet produced antibodies. During inflammation, the concentration of CRP is usually highly increased, and it binds to negatively charged sugar residues and other phosphorylcholine structures like the C-polysaccharides on the cell membrane of pneumococci. Activation via CRP proceeds to the C3 level. CRP also binds factor-H, which inactivates C3b to the form iC3b. Phagocytes have receptors for the iC3b molecule and the Fc-receptor CRP. This enhances the phagocytosis of these targets, and CRP is thought to participate in the clearance of the damaged cells (2, 4, 12).

Lectin pathway

The lectin pathway is analogous to the classical activation pathway, except that it uses different recognition proteins. Activation occurs via mannose binding lectin (MBL), which binds Ca^{2+} dependently to multiple mannose or N-acetylglucosamine sugar residues mainly on the surface of certain microbes, like yeasts. MBL has a high structural resemblance with C1q, but instead of C1r and C1s it binds to mannan binding lectine serine peptidase 1 (MASP1), -2 and -3. MASP1 and MASP2 are serine esterases splicing C4 and C2 and MASP1 as well as C3 molecules directly. The MBL concentration in blood is very low, and the exact role of this lectin for immune defence system is yet unknown (2, 12)(I, II).

Alternative pathway

The antibody independent activation of the complement system is called the alternative pathway (AP). It is triggered by the structures on the microbe surface like polysaccharides, immunoglobulin aggregates and artificial surfaces like the dialysis films in kidney dialysis machines. The AP is activated after pathogen invasion to our tissues or when blood or tissue is in contact with artificial surfaces. Like in other activation pathways, the main assignment is the cleavage of the C3 molecule (Figure 1). The C3 convertase in AP is C3bBb in which Bb is an esterase enzyme resembling C2a. C3b participates in the enzyme complex with C3 as a substrate. The first C3b molecule is formed in the spontaneous hydrolysis of C3 and the formation of C3 (H_2O), which is continuously turned on in a small scale. C3 has reacted with water, but C3a has not yet been detached. C3(H_2O) then participates in the formation of the early state C3 convertase, C3(H2O)Bb, causing a continuous small scale activation of C3. Using this “idling”, the AP system patrols the surfaces in touch with the plasma, ensuring a fast response when the foreign surface is about. Activation proceeds only on the surfaces recognized as foreign by the immune system. AP is also activated during the CP activation; however, this requires that the surface favours AP activation (2, 12) (I, II, III).
Along with the detachment of C3a, the thioester bond is attached to C3b, and the free carbonyl group covalently binds to the hydroxyl groups on the surface and, if the suitable surface is not in the vicinity, C3b is rapidly inactivated. This is also the case when C3b attaches to the host’s own structures. The inactivator is factor I in plasma. Factor H and some membrane regulators act as a cofactor to factor I and, if these cofactors are not properly bound to the surface, C3b is not

inactivated and the surface favours the AP activation. C3bBb complex is formed, which leads to the cleavage of the C3 molecule and thus to the formation of more C3 convertases. The efficiency of the AP system is based on its ability to amplify its own activation. In a matter of minutes, AP can roughly differentiate between self and non-self structures (2, 4, 12).

Other functions of the complement system

Anaphylaxis and chemotaxis

The most important functions of the complement system are outlined in Table 1. The small peptide residues of the complement system (C2b, C3a, C4a and C5) are released during complement activation. C2b is thought to increase the permeability of the small vessels near the site of the inflammation. C3a and C5b are strong anaphylatoxins that cause the mast cells to secrete histamine and other transmitters into the vicinity without IgE assistance. The effects of anaphylatoxins are transmitted via receptors on the target cells (C3aR and C5aR). C5a is a powerful chemotactic agent, oozing out from the complement activation site to the blood stream and luring neutrophils to this site by creating a concentration gradient then utilized by these phagocytic cells. C5a molecules also activate neutrophils (2, 12)

Table 1: The most important functions of the complement system (2, 12).

Functions of the complement system
Lysis of the target microbe (MAC)
Opsonization (C3b, C4b, C1q)
Inflammation transmitters (C5a, C3a, C2b, C5b – 9)
- activation of mast cells to secrete anaphylatoxins
- contraction of smooth muscles
- increase of the permeability of the blood vessels
Chemotaxis and the activation of phagocytes (C5a)
Processing of the immune complexes and molecule aggregates (C3b, C4b, CR1)
Elimination of damaged cell structures (apoptotic and necrotic cells)
Enhancement of the adaptive immune response

Opsonization

Opsonization is a mechanism to enhance phagocytosis by coating the target with the complement components and antibodies attaching to the phagocyte receptors, facilitating ingestion and degradation. There are receptors for C1q, C3b, iC3b and C4g fragments on the phagocytic cell surface, and it has been proposed that opsonization would actually be a more important assignment for the complement than bacterolysis. AP efficiently covers the surface of the target with C3b and iC3b molecules, and pathogens capable of resisting complement activity are usually equipped with the ability to distract the complement activation at C3 level.

In the case of larger parasites, the C3b receptors on eosinophilic granulocytes recognize these C3b molecules attached to the target surface, which activates the immune response (2, 12) (V, VI, VII).

Waste disposal functions

Immune complexes are formed during reactions of antibodies with microbe components, components from foodstuff or from our own decaying cells. Larger aggregates of these complexes have a tendency to sediment. Complement components C4b and C3b prevent this process. Complexes covered with these molecules attach to neutrophil receptors and are directly phagocytized or attached to receptors of erythrocytes to be transported to the liver or the spleen to be destroyed. The same procedure applies for waste products of the host's own cells. C1q has the ability to recognize phospholipids, otherwise present inside the cell but twisted outside with the "flip-flop" mechanism in damaged and apoptotic cells, leading to the phagocytosis of these cells (2, 12).

Enhancement of adaptive immunity

The complement system covalently attaches C3b molecules to invaders. After this, C3b is proteolytically degraded to iC3b and further to C3d. Dentritic cells and B lymphocytes have receptors for these molecules (CR1/CD35 and CR2/CD21). C3b/d coating facilitates the transportation of antigens by dentritic cells to lymph nodes and further presentation to the B cell antigen receptors, augmenting the antibody production (2, 12).

Regulation of the complement system

Uncontrolled activation of the complement activation system is a potential hazard to the host, and it is subjected to rigorous regulation. The prevention of excessive activation is carried out by the lability of the activated combining sites of the reactions and by time and temperature dependent dissociation of some of the active complexes.
The C1 inhibitor (C1INH) is an important factor controlling the classical (C1s and C1r) and lectin pathway (MASP1 and MASP2) esterase enzymes. It mainly prevents the excessive activation of C1s and prevents the auto-activation of C1r. C1s can activate uncontrollably in C1INH deficiency and cause the excessive activation of the CP.
C4bp and factor H conduct analogous roles in classical and alternative pathways, respectively. C4bp degrades C4bC2a convertase and factor H C3bBb convertase, both co-factors for factor I (the inactivator of both C3b and C4b). The role of factor H is to prevent the excessive consumption of soluble complement components and to protect the host's own cell surfaces against amplified alternative pathway activation. Negatively charged structures like glycosaminoglycan and sialic acid on the surface of our own cells assist in the binding of factor H, leading to the inactivation of C3b and thus halting the complement activation. Bacterial surfaces lack these structures, enabling C3b to bind freely (2, 12).
C3 activation is the main target of complement activation regulation, emphasizing the importance of this machinery. The decay-accelerating factor (DAF) molecules on the cell surface degrade both C3/C5 convertases. Membrane cofactor protein (MCP) acts as a cofactor for factor –I (2, 12).
Mac inhibitory protein CD59 attaches to C8 and C9 molecules in C5b – 8 or C5b – 9 complexes preventing the further polymerization of C9 molecules and further reduces the killing effect of MAC.
Properdine (P) prevents the dismantling of the C3bBb convertase and stabilizes the alternative pathway C3/C5 convertase attached to the activating surface, thus enhancing the amplification cascade (2, 12)

Common characteristics of complement components

There are structural similarities between the different activators and regulators of the complement system. Enzymes like C1r, C1s, MASP1, MASP2, C2, B, D and I are all serine esterases. Pairs such as C1s-C1r and C2-B are constructed out of similarly structured molecules, probably evolved trough the duplication of genes during the evolution. Genes encoding components C2, B and C4 are located in the human chromosome 6 (the same chromosome includes MHC genes), and both C2 and C4 have a huge genetical variety (2, 12).
The genes of components C3, C4 and C5 are located in different chromosomes, but they are very similar in structure. The components participating in the formation of MAC, C6, C7, C8 and C9 also have high structural resemblance, which is thought to be connected to the ability to associate with each other (2, 12).

2.1.3 Phagocytosis

Introduction

Some leukocytes are able to "swallow" invading microbes in an active and receptor-mediated process called phagocytosis. Vacuoles and granules inside these cells contain myriads of antimicrobial molecules and systems, ready to destroy the target after ingestion. In addition to microbial killing, phagocytic cells also discard the waste material, taking on an important role in normal household and cleaning duties, like the removal of the host's own old, apoptotic and necrotic cells (3, 4, 13) (V, VI, VII).
The most important phagocytes include granulocytes (polymorphonuclear neutrophils [PMNs] and eosinophils) and mononuclear phagocytes (monocytes, later maturing to macrophages). Granulocytes are efficient killers rapidly phagocytizing their targets. Some intracellular microbes, including some bacterial strains and protozoa, can resist phagocytosis and survive. Macrophages are matured from monocytes in different tissues, usually outside the circulation, and they are specialized against intracellular antagonists, being more efficient in this role than neutrophils. Some intracellular bacteria, like the strains of *mycobacteria*, can survive inside the macrophage and cause severe infections (4, 10).
In order to form the functional phagocyte response and eliminate microbes, the phagocytic cells have to be lured to the site of inflammation with chemotactic transmitters and be activated. This happens through their target recognition receptors. The ligand can be a structural molecule on the microbe surface or antibody or a complement component attached to surface.
When a phagocyte has seized its target and attached to it, the active process of ingestion starts. The leukocyte crawls and reaches out around the target cell, finally forming a bag enclosing the target. Intracellular actine and myosine filaments, also forming the cytoskeleton of the cell, provide the movement. Energy is derived from anaerobic glycolysis. The vacuole formed inside the cell is designated as phagosome. The degradation of the target starts when different granules, vacuoles and lysosomes, containing different antimicrobial systems are fused into the phagosome forming the entity called phagolysosome (14-17).

Neutrophil function

The PMNs, or granulocytes, comprise approximately 50–60 % of the circulating leukocytes in humans, being the most abundant leukocyte group in mammalian peripheral circulation. Neutrophils form the first line of defense in the innate immune protection by phagocytizing and digesting the targets, like bacteria and fungi, as well as biological and mechanical waste products. They are the key effector cells in the host defense response against microbial invasion, providing rapid deployment and the effector arm of the immune system (18-23) (V, VI, VII).
Peripheral neutrophils are end-stage cells, and once released from the bone marrow, they circulate with a half-life of 6–20 hours before losing functional capability and leaving the body in a random (non-age-related) fashion. Their survival in tissues under steady state conditions is about 4–5 days. About 10^{11} neutrophils enter and leave the body daily. Both mature and bone marrow neutrophil pools are called into play during an acute infection (10). In bloodstream and extravascular spaces, neutrophils exert their antimicrobial effects through complex interaction with proinflammatory cytokines like interleukin 8 (IL-8), tumor negrosis factor α, IL-1a, interferoni γ and granulocyte-macrophage-colony stimulating factor, produsedby other immune cells (macrophages, dentritic cells and lymphocytes), antibodies, complement, and chemotactic factors.
The mature neutrophil is primarily a phagocytic cell with different types of granules containing antimicrobial agents. The main types are azurophilic granules, containing myeloperoxidase (MPO), hydrolases, lysozyme and a number of cationic proteins possessing antimicrobial activity, and secondary granules with lactoferrin and some lysozyme. In the mature neutrophil, 80–90 % of the granules are azurophilic and the rest are secondary granules (17, 23, 24).
Phagocytosis by neutrophil is composed of 4 interrelated phases: chemotaxis, opsonization, ingestion, and killing (25).

Recruitment of phagocytes into the site of inflammation

Circulating neutrophils are concentrated in areas of inflammation because of the increased affinity of endothelial surfaces, leading to attachment to the capillary wall and migration to the tissues. Cytokines and expecially chemokines (a subclass of cytokines) like IL-8 (the neutrophil chemotactic factor) produced by macrpphages and epithelial cells, and formyl-Met-Leu-Phe, a peptide produced by bacterial cells, recruit neutrophils and other leukosytes into the site of inflammation (26). Chemotaxis can be defined as the ability of motile cells to recognize and respond to a suitable chemical gradient with directional migration. Both factors influence the accumulation of inflammatory cells at an infective focus (4).
When the complement system is activated and C5a-polypeptide is released into the circulation. As a very powerful chemoattractant it initializes the recruitment of phagocytic cells to the site of infection and APC cells to the lymph nodes (4).
Bacteria in the site of inflammation elaborate chemotactic signals like peptides, with formylmethionine as an N-terminal residue. This structure is the initiation amino acid in bacterial protein synthesis, totally lacking from eukaryotic cell protein synthesis, a certain marker for bacterial metabolism in the host's systems (4).
Phagocytic cells have high-affinity receptors for chemoattractants. Ligand binding leads to intracellular signal transduction, resulting in increased motility and target attachment. As a consequence, the phagocytic cell activates the mechanisms essential in phagocytosis and killing. The moving neutrophil has a specific, dynamic morphological feature caused by actin and myosin filaments. Along the other structural proteins in the cell, they provide the structural support and dynamic forces needed for cell movement in the correct direction (27-29).

Attachment to the microbe

Phagocytes can recognize and attach to their targets by directly recognizing either the structures of the cell surface or specific molecules, opsonins (Greek *opsonein* to prepare food for), attached to the surface, like complement components and antibodies. The function of opsonins is to react to microorganisms and make them more susceptible to ingestion by phagocytes. The virulence of many pathogens relates in part to their ability to evade phagocytosis by virtue of certain surface antigens. Microorganisms with the antiphagocytic surface factors include pathogenic species like *Streptococcus pneumoniae*, group of B *Streptococci*, *Klebsiella pneumonia* and *Bacillus anthracis.* A large variety of receptors recognizing different microbe antigens and opsonins are required in the process of phagocytosis.

The recognition is done by the lectin like structures in the phagocyte receptors, recognizing sugar residues like galactose, mannose, beta glucan, lipoprotein or lipopolysaccharide (LPS). (30-34).

Immunostimulants, the surface molecules on the microbes, typically form repeating structural patterns (PAMP), recognized by the pattern recognition receptors (PRR) including complement proteins in blood and toll-like (TLR) and nucleotide-binding oligomerization domain receptors (NOD) (2, 4, 35).

TLR are transmembrane proteins found in both the plasma membrane and intracellular membranes, especially expressed by phagocytes like neutrophils, macrophages and dentritic cells. The immune response in mammalians is usually triggered by TLR. They recognize the common products of the microbes, like LPS and bacterial DNA. Activation trough TLR triggers a signal transduction cascade leading to the transcription of genes required in immune responses.

Similar to TLR in function but with different specificity to ligands are the intracellular NOD, which recognize the bacterial cell membrane particles (2).

A Glycosylphosphatidylinositol (GPI) linked membrane protein CD14 forms a major binding site for LPS. The bindings of the LPS to the CD14 trigger a formation of CD14/CR3 complex, reported to dissociate after neutrophils attach to their substrates. It has also been reported that CD14 affects LPS mediated signaling in leukocytes through the formation of TLR 4/CD14 (2, 36).

Because some bacteria twine inside protective capsules, protecting them from direct recognition by phagocyte receptors, the complement system and antibody mediated attachment of phagocytic cells plays a more important role.

Neutrophils have receptors for Fc- part of antibodies. FcγRI III recognizes IgG class antibodies and FcαR IgA class antibodies on the target surface.

The most important complement receptors (CR) participating in phagocytosis are CR1 (CD35) recognizing C3b (and C4b) and CR3 (CD11/CD18) recognizing iC3b. CR1 is also present in plasma in a soluble form (sCR1). There is also a receptor for C1q component (C1q receptor or Collectin receptor) recognizing collagen-like structures in C1q and in other similar collectins like MBL in serum (2, 4, 30-34).

On the human cell surface, mannose residues are covered with other sugars, and mannose receptors, in principle, only recognize bacterial cells by uncovered mannose structures on their membranes.

The efficiency of phagocytosis is increased when the target cell is opsonized with several different factors. Different opsonins attach differently to their targets. C3b binds covalently with the thioester bond and IgG noncovalently. C3b is rapidly inactivated to form iC3b, indicating a more vital role for CR3 in phagocytosis, whereas CR1 has a more important task in collecting and transporting targets covered with C3b into the vicinity of the phagocytic cell (37).

Since the alternative complement pathway is present in nonimmune individuals and is not dependent on the presence of the anti-capsular antibody for its action, it has been considered to play an important role in critical stages of infection prior to the production of a specific antibody. This is also the stage where surface phagocytosis is considered the most important. In surface phagocytosis,

encapsulated bacteria are trapped between leukocytes and tissue surfaces or along with leukocytes in the interstices of the fibrin cloth (3, 10).
In the addition of alternative complement pathway activation, collectin binding also enables target opsonization in primary encounters.

Ingestion

Once a particle or microorganism has been adequately opsonized, the machinery of cellular membrane locomotion is activated. Since energy for this process is provided by anaerobic glycolysis, phagocytosis can take place even in the anaerobic interior of an abscess. The movement of pseudopodia (the temporary cellular projections of neutrophil) around microorganisms requires actin and myosin microfilament activity. Ingestion requires interaction between opsonic ligands distributed homogeneously over the surface of the particles to be ingested and the receptors on the phagocyte membrane. This sequential interaction results in the circumferential flow of the phagocyte membrane around the particle, "zipping it up". Following ingestion, the particle is invaginated by the cell membrane forming phagosome. Cell pseudopodia fuse on the distal side of the material being ingested and the particle becomes encased within a phagocytic vacuole (phagosome). The phagosome buds off from the cell periphery and moves towards the center, apparently through the mediation of microtubules (3, 4). Shortly thereafter, lysosomes fuse with the phagosome to form a structure called the phagolysosome. Degranulation is the process of fusion of lysosomes and pahgosomes with the subsequent discharge of intra-lysosomal contents into the phagolysosome.

Respiratory burst

The term respiratory burst (RB) describes a metabolic pathway, dormant in resting cells, the function of which is to produce a group of highly reactive antimicrobial agents by the partial reduction of oxygen. RB occurs with any perturbation of the cell membrane of the neutrophil and is independent of the process of ingestion (14, 16, 17, 23, 38-40) (VI, VII). There are four components of RB: the increase in oxygen consumption, superoxide ($O_2^{-\cdot}$) production, H_2O_2 production, and hexose monophosphate shunt activation (Figure 2). The mechanism of the RB is as follows: (1) Contact with a particle with the neutrophil membrane activates a membrane-associated flavoenzyme system currently referred to as NADPH oxidase. (2) NADPH oxidase catalyses the one-electron reduction of oxygen (O_2) to superoxide ($O_2^{-\cdot}$), using NADPH as an electron donor. Two superoxide molecules combine in the presence of the enzyme superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2):

$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \rightarrow O_2 + H_2O_2$$

(3) Myeloperoxidase (MPO) is deposited by degranulation into phagocytic vacuoles, where it, in the presence of H_2O_2 and halide, catalyses reactions leading to the formation of further reactive oxidants such as hypochlorous acid (HOCl) (Figure 2). MPO is a peroxidase enzyme claiming to play a fundamental role in the oxidant production and thus in the antimicrobial activity of neutrophils (17, 23, 38, 41-46).

$$H_2O_2 + Cl^- \rightarrow HOCl + H_2O$$

(4) Superoxide that escapes from the phagocytic vacuoles is reduced to H_2O_2 at an enhanced rate by SOD. Cytoplasmic H_2O_2 is detoxified by the catalase and glutathione peroxidase-glutathione reductase system. Both the superoxide-forming NADPH oxidase and the glutathione system generate

$NADP^+$ in the course of their activity. $NADP^+$ is converted back to NADPH by the hexose monophosphate shunt (3, 47).

Degranulation

The destruction of microorganisms by neutrophils is intimately associated with the process of degranulation, the release of the granule contents into the phagosomes. There is also firm evidence that degranulation takes place in the cell exterior.
Multiple factors like proinflammatory cytokines, during the immune response, physical stress, temperature changes, some antigoagulant types and isolation can trigger the neutrophil degranulation (32). Degranulation is an active process and requires energy expenditure by the cell and thus an impairment of the normal metabolic pathways of the neutrophil (Figure 2). Especially the oxygen consumption of glucose through the hexose monophosphate shunt interferes with degranulation and subsequent intracellular killing (48-50).
There are a variety of classifications for different neutrophil granules and vesicles. According to Faurschou and Borregaard (2003), human neutrophils contain 3 major types of granules and furthermore secretory vesicles. Primary granules (azurophilic granules) contain abundant hydrolytic lysosomal enzymes, large amounts of MPO, lysozyme, defensis, and serine proteases elastase and cathepsin G (48-53).
Secondary (specific) granules appear as the promyelocyte matures to a myelocyte in the bone marrow. They are smaller than primary granules, stain poorly (neutrophilic), and later outnumber primary granules in the mature neutrophil. Secondary granules contain NADPH oxidase, alkaline phosphatese, lysozyme and collagenase. The third type of granules, also referred to as tertiary granules, contain gelatinase and cathepsin (48-50).
As phagollysosome is formed after microbial engulfment, neutrophil granules undergo violent movement in proximity to the phagolysosome, fuse with the phagocytic vacuole, and disappear from the cytoplasm (degranulation). Somewhere between the stages of attachment and digestion the membranes of specific granules are fused with the phagolysosomes. As a result, lactoferrin and lysozyme are allowed to enter the extracellular space. Azurophilic granules fuse slightly later. As with chemotaxis and ingestion, microfilaments and microtubules appear to be important for the fusion of phagoslysosomes and granules to occur (48-50).

Killing

The mechanisms by which neutrophils kill microorganisms are not fully understood. However, it is clear that multiple interlocking microbial systems are present. The subject is controversial and under continuous debate (14-17, 25, 54). According to traditional textbooks, the antimicrobial mechanisms of human PMN can be divided into two broad categories: oxygen-dependent and oxygen-independent (Table 2) (3, 4, 10).
A. Oxygen-dependent systems: Oxidative mechanisms can be divided into MPO-mediated and MPO-independent systems. The MPO system has been proven to participate in oxygen-dependent antimicrobial processes in phagocytes (14-17). However, studies have revealed that up to 0.025 % of the general population has total or subtotal MPO deficiency, meaning neutrophils do not contain MPO, and yet these individuals do not exhibit a predisposition to infections (17). Furthermore, macrophages do not produce MPO and they prominently kill microorganisms. The primacy of MPO in oxidative killing is open to questions (4).
Oxygen-dependent but MPO-independent killing clearly takes place, although the mechanisms are still under wide dispute. The established views are challenged by suggestions that the main role of NADPH-oxidase is not the production of superoxide (Figure 2) but the enabling of the solubilisation

of elastase and gathepsin G by increasing osmolarity by the polarization of the phagolysosomal membrane, allowing higher influx of potassium ions (25, 54).

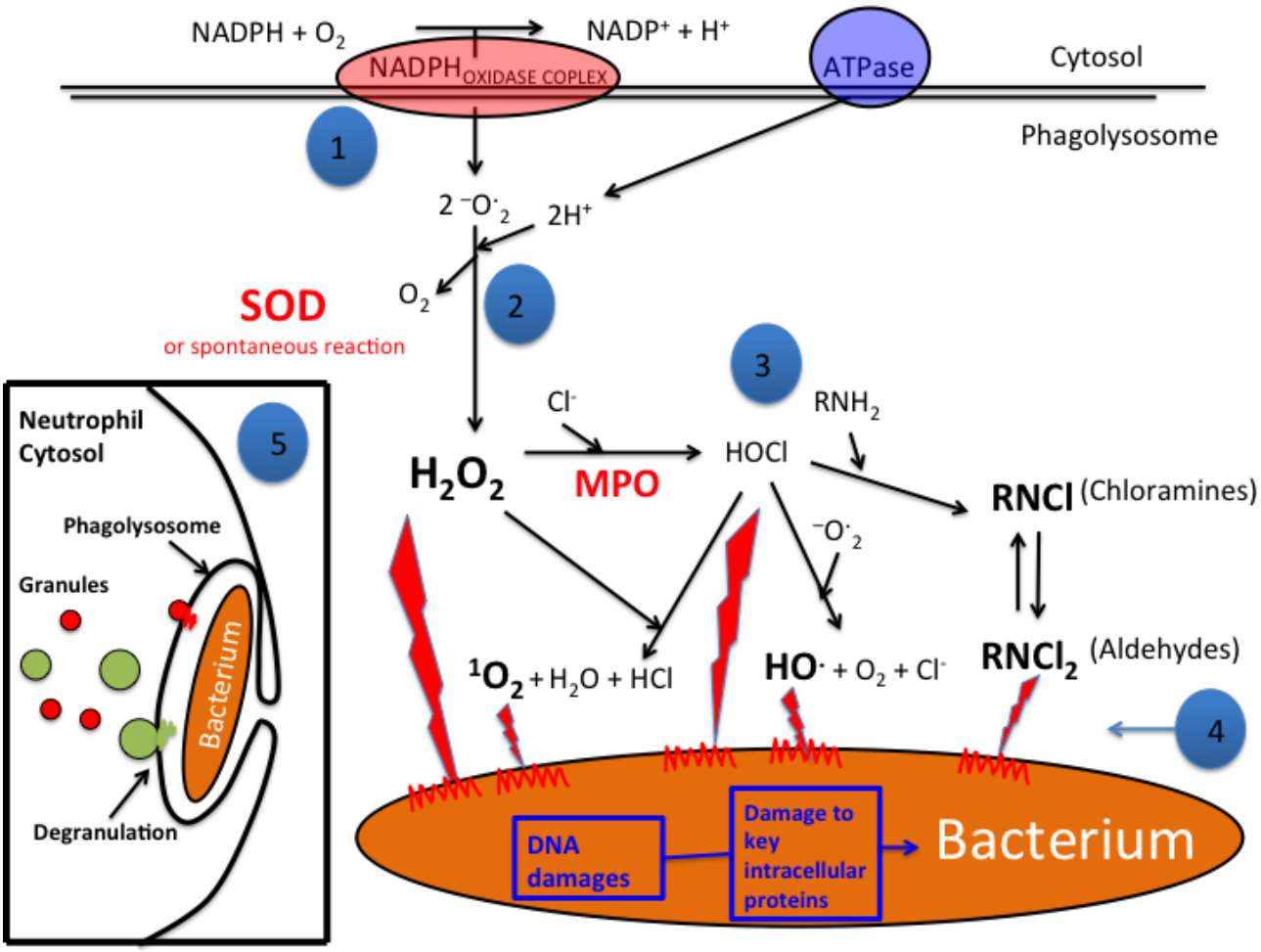


Figure 2: Simplified representation of phagocytosis. The figure shows the respiratory burst and oxygen-dependent killing reactions (55). (1) NADPH oxidase function, (2) Production of H_2O_2 from superoxide, catalysed by the superoxide dismutase (SOD), (3) Myeloperoxidase (MPO) reaction, (4) Oxygen-dependent killing, (5) Principle of phagocytosis.

The second theory, also followed in this book, is that because actual bacterial killing takes place in phagolysosome at increased pH (pH near 8), the chlorinating activity of MPO would be absent and, moreover, the H_2O_2 concentration in phagolysosome would be high enough to support the bacterial killing (VI).

Oxygen-dependent killing

1. MPO-mediated systems

As mentioned in the previous section, the importance of MPO in bacterial killing in phagocytes is controversial and to date this has not yet been clarified. MPO is found in the azurophilic granules of neutrophils, and it is the most abundant protein produced by these cells, counting up to 5 % of their dry weight. In conjunction with the H_2O_2, the oxidizable halide factor, and the acidic pH, a potent

antimicrobial system is developed in the pahgolysosome, which has antibacterial, antifungal, antiviral, and antimycoplasmal capabilities.
Seymor J. Klebanoff first reported the system in 1967, and it has been widely confirmed since that the incubation of bacteria with H_2O_2, MPO and halide, like Cl^- ion in acidic or neutral pH results in efficient killing at nanomolar scale H_2O_2 concentrations (VI). The killing capacity is dependent on the number of bacterial cells used in the reaction. In the same circumstances without MPO, similar killing is achieved with tenfold H_2O_2 concentration. MPO catalyzes the oxidation of halide ions to hypohalide ions by H_2O_2 (Figure 2 and 3) (15-17, 23, 38-40)
There are a number of potential mechanisms whereby "activated halide" might damage microorganisms, including the halogenation of the bacterial cell wall, the decarboxylation of amino acid with the release of toxic aldehydes, and production of singlet oxygen (Figure 3).

Table 2: The antimicrobial mechanisms of human polymorphonuclear neutrophils (PMN) can be divided into two broad categories: oxygen dependent and independent

Antimicrobial Systems of the PMN

Oxygen-dependent

MPO-mediated

- MPO
- H_2O_2
- Oxidizable cofactor (usually a halide)

MPO-independent

- H_2O_2
- Superoxide anion
- Singlet oxygen
- Hydroxyl radical

Oxygen-independent

- Cationic Proteins
- Lactoferrin
- Lysozyme
- pH
- Nuclear histone
- Elastase
- Gathepsin G

Under ordinary circumstances, the H_2O_2 utilized in this system is considered to arise from the RB. With deficient H_2O_2 production (eg. chronic granulomatous disease [CGD], a genetic disorder leading to the total deficiency of NADPH-oxidase), certain microorganisms may paradoxically provide the H_2O_2, which mediates their own killing (10). Among these are *pneumococci* and other *streptococci*. These bacteria are classified as *Lactobasillaceae*; their terminal oxidations are catalyzed by flavoproteins, which reduce oxygen into H_2O_2. Because they lack catalase, H_2O_2 accumulates and can be used in killing reactions. This may explain why patients with CGD show no particular predisposition to *streptococcal* infections (2, 10).
Chloride is present in the neutrophil at a level considerably above that required for participation in MPO-mediated reactions. The concentration in phagolysosome is reported to be 70 mM or less (24, 56). Chloride could enter the phagocytic vacuole along with the phagocytosed particle or could be transferred across the membrane of the phagolysosome. HOCl is widely recognized as a highly

effective antimicrobial agent (Klebanoff 1968, Dettenkofer, Hauer & Daschner 2004, LeChevallier, Cawthon & Lee 1988, Kaatz et al. 1988). Bromide (35 µM in plasma) can also participate in the formation of the hypohalide acid (57). Another logical halide participant could be iodide, provided either directly from serum (serum concentration < 0.8 nM) or by the deiodination of the thyroid hormone, membrane bound in both neutrophils and bacteria (57).
The conventional view is that the MPO-H_2O_2-halide system would be essential in killing because of the surplus amount of MPO present and because the formed hypohalide is a powerful antimicrobial agent. The MPO-H_2O_2-halide system killing is pH-dependent (optimum at pH 5.0–6.0), and the peroxidase activity of MPO is halted at higher pH values (pH 7.7–8.0)(58-60). During phagocytosis, the pH in the phagolysosome first increases and then gradually decreases. In human neutrophils, the increase to 7.8–8.0 takes place during the first 20 to 30 minutes due to the fact that the dismutation of suoperoxide anion to H_2O_2 consumes H^+ during the RB (25, 61-64). This is one piece of evidence to support the theory that the MPO-mediated system is not responsible for the actual killing (VI).

Singlet oxygen ($^1O_2$) has the same molecular formula as the atmospheric (triplet) oxygen (O_2), but it differs in the distribution of electrons around the two oxygen nuclei. There are several potential sources for singlet oxygen during RB:
1. OCl^-, a product of the MPO reaction, is known to react with H_2O_2 to produce singlet oxygen:

$$OCl^- + H_2O_2 \rightarrow {}^1O_2 + Cl^- + H_2O$$

2. The reaction of superoxidase ($O_2^{-\cdot}$) with H_2O_2 can produce singlet oxygen and hydroxyl radical. This reaction (Haber-Weiss reaction) is strongly catalyzed by iron and lactoferrin.

$$O_2^{-\cdot} + H_2O_2 \rightarrow {}^1O_2 + OH^\cdot + OH^-$$

3. Spontaneous dismutation of superoxide (in the absence of superoxide dismutase) may produce singlet oxygen.

$$2O_2^{-\cdot} + 2H^+ \rightarrow {}^1O_2 + H_2O_2$$

The lifetime of singlet oxygen is short, with the dissipation of energy by light emission, thermal decay and chemical reaction. Singlet oxygen has a particular propensity to react with double bonds and would be lethal to any biological system that is contacted. Among other oxidizing species in neutrophil, like H_2O_2 and $O_2^{-\cdot}$, singlet oxygen can also produce a low level burst of light (chemiluminescence [CL]) by phagocytizing PMNs. The emission of light from the H_2O_2 and $O_2^{-\cdot}$ reaction products occurs at all visible wavelengths. In contrast, the singlet oxygen transition to atmospheric oxygen produces a characteristic red light emission. The standard tests of leukocyte CL reflect only a nonspecific accompaniment of the RB and do not prove the presence of singlet oxygen (3, 10, 14, 16, 17, 23, 38-40).
Numerous individuals have been identified with MPO-deficient PMNs. The bactericidal activity of MPO-deficient PMNs is characterized by a lag period, but the killing of the majority of the ingested microorganisms is eventually complete. Obviously, other mechanisms for killing the invading pathogen exist.

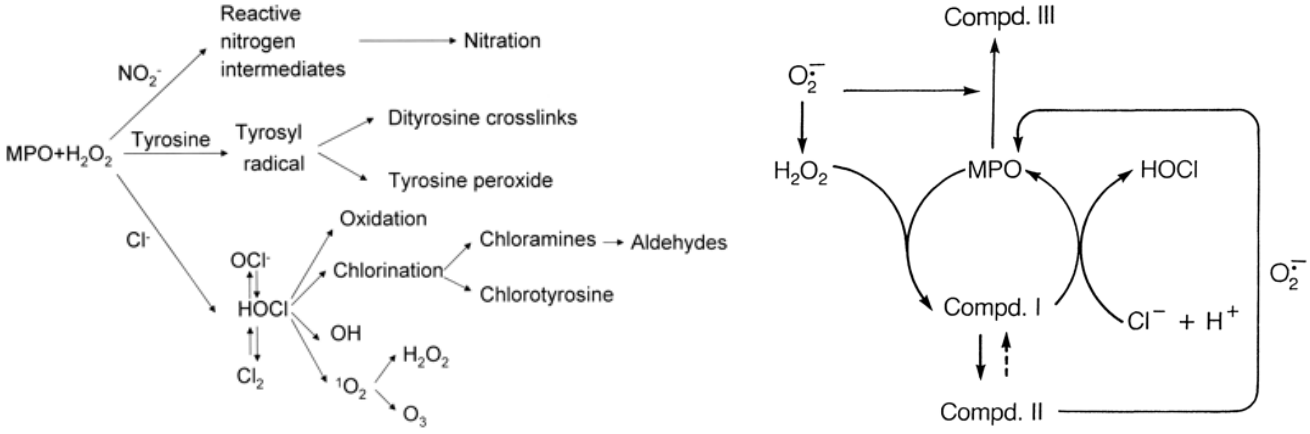


Figure 3: MPO complex formation (left) and products of the MPO-mediated antimicrobial system (right) by Klebanoff (23, 39).

2. MPO-independent systems

At high enough concentrations, H_2O_2 has direct antimicrobial activity. In addition, it may act in concert with ascorbic acid and certain metal ions to kill ingested microorganisms by nonenzymatic means. During the actual killing in the phagolysosome the pH has actually increased to near pH 8, where the MPO chlorination activity is absent and is no longer enhancing the killing. We have shown that H_2O_2 concentration in phagolysosome alone, without MPO, is able to kill one bacterial cell (VII) (60). Some microorganisms are more susceptible to H_2O_2 than others, depending on their ability to produce catalase or peroxidase (3).

A direct toxic effect of superoxide anion on microorganisms has been postulated. However, many microorganisms contain superoxide dismutase and would be expected to resist the action of this radical in phagolysosome. Actually the H_2O_2 formed from superoxide anion has greater microbicidal potential when employed as a component in the MPO-mediated system. Superoxide anion is only weakly bactericidal (14-17, 38, 46).

Singlet oxygen is reportedly present in reduced amounts in MPO-deficient PMNs. It is possible that the radical comes about as a by-product in the spontaneous dismutation of superoxide (14-17, 38, 46).

Hydroxyl radical, formed by the interaction between the H_2O_2 and the superoxide anion, may be formed in the leukocyte by the Haber-Weiss reaction. Alternatively, superoxide may have to react with an intermediate peroxy-compound before $OH^{\cdot}$ is produced. The hydroxyl radical is a highly unstable oxidizing species that reacts almost instantaneously with most organic molecules that it encounters. It is best known as one of the major products resulting from the passage of ionizing radiation through water and has been implicated in the toxicity of radiation for tissues (14-17, 38, 46).

Systems of microbicidal activity that are independent of the RB (Figure 8) include those based on cationic proteins, lactoferrin, lysozyme, nuclear histones, elastase, and production of acid in the phagolysosome. Nonoxidative microbicidal mechanisms are of obvious importance, because ceratin microorganisms can clearly be killed by neutrophils under anaerobic conditions (eg. *Pseudomonas aeruginosa, Candida parapsilosis*, and anaerobic bacteria) (3).

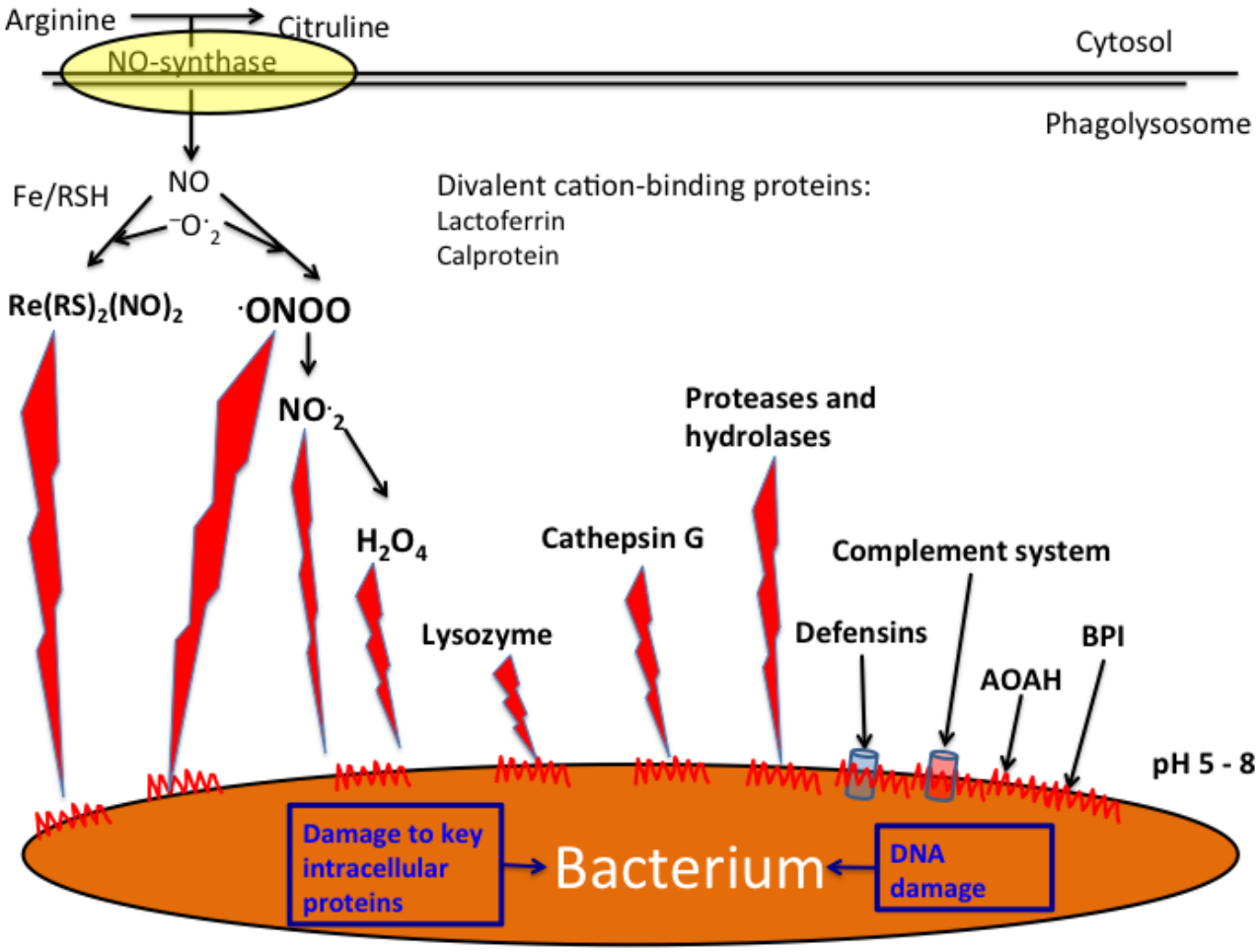


Figure 4: The schematic diagram illustrating the other mechanisms used by the phagocytes to kill bacteria. AOAH = acyloyacyl hydrolase. BPI = Bactericidal/Permeability-increasing protein

Oxygen-independent killing

Cationic proteins from azurophilic granules, like defensins, were the first antimicrobial substances defined in neutrophils. Cationic proteins are rich in arginine, they are the most effective at neutral pH and they rapidly affect the ability of microorganisms to replicate without destroying their structural integrity. Separate cationic protein subclasses demonstrate specificity for certain classes of microorganisms. The susceptibility of gram negative (gram (-)) microorganisms to lysis by neutrophils is related to the amount of polysaccharide in the outer cell envelope; smooth strains may be more difficult to kill than rough strains (3, 48-51).
Lactoferrin from specific granules may exert an antimicrobial function by binding and withholding required iron from ingested bacteria and fungi that depend on iron for their existence. Lactoferrin is also discharged into the extracellular medium during phagocytosis so that lactoferrin may exert a substantial proportion of its effects outside the phagocyte. Impaired bacterial killing by neutrophils can be associated with lactoferrin-deficient neutrophil granules. (3, 48-51).
Lysozyme, present in both primary and specific granules, damages bacterial cell walls by catalyzing the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan. Bacteria intrinsically insensitive to lysozyme become sensitive to it in

the presence of the complement or ascorbic acid and H_2O_2. Because bacterial death often precedes the action of lysozyme, this enzyme may serve in a digestive rather than microbicidal capacity (3, 48-51).
After RB (60 min after ingestion), pH in phagolysosome falls rapidly to pH 4.5–5.0. Acidification appears to enhance the function of some antimicrobial enzyme systems, like MPO-H_2O_2-halide and lysozyme. As mentioned above, the actual killing chronologically precedes the acidification, so its role as an MPO system activator is adverse.
The ideal pH for cationic proteins is neutral or slightly alkaline. Some bacteria are killed directly by organic acids in an acidic medium or by lipophilic acids.
These substances are released into surrounding tissues after death and autolysis of the cells, and they possess direct antimicrobial activity (3, 48-51).
Granulocytes contain a variety of proteases that are active at neutral pH (eg. cathepsin G, serine esterase, elastase). These substances may be more important for antimicrobial reactions and ingestion than has previously been considered (3, 25, 48-51).
As noted previously, serum factors promote the phagocytic killing of microorganisms by their opsonic function. The active complement system is still operating on the bacterial cell surface during the ingestion and in phagolysosome, augmenting the bacterial destruction (2, 3).

Extracellular release of granulocyte constituent

RB is activated by the perturbation of the neutrophil membrane independently of the process of ingestion. Moreover, specific granules may discharge their contents outside the cell during phagosome formation. The fungal mycelial element, clearly too large to be completely ingested by human neutrophils, can still be killed by a process that includes the spread of neutrophils over the mycelial surface, RB activity with iodination of the fungal surface, and partial neutrophil degranulation.
Exocytosis of granulocyte contents is apparently a common phenomenon and may play a more important role than merely the killing of attached, uningested microorganisms. For example, the MPO-halide system is capable of inactivating a variety of chemotactic factors (C5a, synthetic chemotactic peptides, and bacterial chemotactic factor), thus modulating the inflammatory response. The same system also inactivates proteinase inhibitors in the locale of the PMNs, thus allowing proteases released from the phagocytes to more readily damage the connective tissue structure.
The oxidative stress caused by inflammation and mediated largely by the intermediates released by neutrophils are claimed to be causative factors in conditions like cardiovascular diseases and diabetes. Especially the extracellular MPO is pointed out to participate in these events (65-70). This same extracellularized MPO can have an important role, since there is a report of macrophages actually utilizing the MPO produced by neutrophils in their bacterial killing (71).

2.2 Bacterial growth

The factors involved in the reproduction of bacteria are both numerous and complex. The complex molecules provided by the medium in which the bacteria are growing are broken down, and from the resulting simpler substances the bacterial cytoplasm and membrane is synthesized. In spite of the different processes concerned, the multiplication of bacteria under favourable conditions tends to take place in a regular manner, and, after inoculating a fluid medium with bacteria, the bacterial number can determine the various stages of growth at regular intervals. By such means it is possible to examine the effect of various factors on the rate and degree of bacterial growth *in vitro*.

Knowledge of the factors influencing bacterial multiplication *in vitro* is valuable, because it throws some light on the development of bacteria *in vivo* (3, 72-79).

An organism, whether being a bacterium or not, has an identity specified by its genetic make-up and largely functioning by the virtue of the enzymes it contains. For the individual and the species to survive and be perpetuated, the specification and the functional system must be maintained and reproduced. Growth can be considered at the level of an individual cell or at that of the population, although the latter depends on the former. When one organism becomes two, everything has been duplicated: the amount of cell wall, of the membrane, of ribosomes, the DNA, the RNA, the proteins, the other cytoplasmic constituents such as the ions, amino acids and intermediates of metabolism. Essentially a bacterial cell increases to double its size and then divides into two (72, 73).

Because of the small size of the bacterial cell, *in vitro* growth is usually investigated by measuring the growth of a larger population of bacterial cells cultured in liquid media. Bacterial growth in the medium is normally determined by using spectrophotometry, determining turbidity "cloudiness" of the medium (80, 81) (II). This means the measuring of the intensity of the light passing through the bacterial cell suspension; the more cells in the suspension, the higher the turbidity and less light passing through the suspension. This optical density (OD) measurement provides real time data from the patch culture growth, but the calibration curve is needed to convert the turbidity reading to a number of bacterial cells in the suspension (80, 81) (II) Moreover, the turbidity does not differentiate between viable and dead bacteria. The viable count, i.e. the number of living bacteria in the medium at a certain time point is traditionally determined by plating a standard amount of the serial decimal dilutions of the suspension. After incubation at the optimal temperature for at least 12 h (often 24–48 h), depending greatly on the species used, the total number of colonies on plates containing a countable number is obtained and an average made after the dilutions have been adjusted. In this manner, the number of living organisms per ml can be estimated with a moderate degree of accuracy. Counts of the total number of cells in a definite volume of the sample can also be defined using flow cytometry or a microscope with different cell counting chambers. These methods, with proper cell staining, can also differentiate between viable and dead cells (80, 81) (II).

The growth curve (Figure 5) of a population culture can be plotted as growth versus time. The Y-axel is usually the number of organisms (in the log scale) but can also be mass or some other index, indicating the growth of the organism in question (72, 73, 80-82).

After seeding the inoculum in fluid media, the growth starts with the increase in cell size without the immediate increase in the cell number when bacterial cells are introduced to the fresh culture medium with proper nutrients. During this lag phase (Figure 5), new essential components, prior to the cell division including ATP, essential cofactors, membrane lipids, ribosomes and DNA are synthetized (72, 73). The lag phase represents the adjustment of the bacterium to changes in the environment. The variations in the length of the lag phase are considerable depending on the nature of the medium and the condition of the microbe. Metabolic activity per cell increases during this period and reaches a maximum value late in this period or early in the next growing phase (72, 73).

The next phase is the stage of the most rapid multiplication, which determined the logarithmic phase (log phase) (Figure 5) or exponential growth phase. During this phase, microorganisms are growing and dividing at the maximal rate possible. The rate of multiplication remains constant. The time taken for the number of organisms in a culture to double is called the mean generation time (72, 73). This is the time required for each bacterium cell to divide into two daughter cells, being the same troughout the generation time. That is, the microorganisms are dividing and doubling in number at a regular interval. The exponential growth rate is determined to a great extent by three factors: the temperature of incubation, the nature of medium and the species of bacteria (72, 73). Generation time varies greatly between bacterial species, as *E. coli* may multiply as rapidly as once every 20 min, while the *tubercle bacillus* has a generation time of many hours (72, 73). In the vigorously growing exponential growth phase, the microbe population is the most uniform and viable in terms of chemical and physiological properties, and this is why a biochemical study usually uses cells from

exponential phase cultures (72, 73). Another reason for this is, especially in the case of antimicrobial analysis, that the cells late in the lag and early in the log phase are generally more sensitive to heat treatment and to the chemical agents than the cells from more mature cultures, a behaviour referred to as the "physiological youth" of the cells, ascribed already in 1923 by Sherman and Albus (72, 73). They also demonstrated that the cells are generally much larger during the early phase of growth than those from the parent culture. At the end of the exponential phase, the rate of multiplication decreases and the average generation time increases. The organism continues to increase in number but at a slower rate (Figure 5).

Indefinite growth in batch culture is not possible, and the log phase is followed by a period in which the viable count remains steady; this is consequently termed the stationary phase where bacterial population has normally attained the level of about 10^9 cells/ml (83, 84). During the stationary period, the net value of the population cell number stays constant (Figure 5), which means that the number of bacteria dying balances the formation of fresh forms or the population remains metabolically active but ceases to divide (72, 73, 82, 85). The reason for the marked decrease in the multiplication of the bacteria growing in the closed system depends on the exhaustion of nutrients, as in the development of each new generation the demand for building materials and energy theoretically doubles. Other reasons for the decrease in the rate of multiplication are the production of noxious metabolic substances and the gradual lack of oxygen in the case of aerobic bacteria (74, 86-88).

Figure 5 illustrates behaviour during the growth of bacteria in a mixed medium with multiple carbon sources, representing the adaptation by the bacteria to environment. *E. coli*, for example, in a glucose-arabinose medium, exhibit a double growth cycle. The curve consists of two exponential phases of growth separated by a phase during which the growth rate passes through the minimum. After a lag, *E.coli* grew in the glucose-arabinose medium until all the glucose has been utilized. A second period of lag was observed, and then growth was reinitiated and arabinose was utilized, the concentration of sugars being such that the extent of growth upon each substrate was limited by this factor. This phenomenon is explained on the basis of one sugar (or other substrate) inhibiting the formation or function of the other and is called diphasic growth (Figure 5) (72, 73). When growth is limited by the exhaustion of the carbon source, it is frequently possible to resume growth more or less immediately by further addition of the substrate – even after the culture has been in the stationary phase for many hours (72, 73, 86). This soon results in a culture that is growing exponentially (Figure 5).

The constitution of the bacterial cell is determined to considerable extent by the medium in which it is growing. A "rich medium" is one that provides many substances, such as amino acids, purines, pyrimides and bacterial vitamins, which would otherwise have to be synthetized. This kind of environment tends to cause cells to grow more rapidly and to be larger than in a poor medium. Merely altering the carbon course from one substance to another may provoke changes in the mean generation time and the size of cells. Sometimes one substrate (e.g. glucose) is preferred to another (e.g. arabinose), and organisms will grow exponentially in the presence of both, using only one until it is exhausted and then, after a short lag, using the other but with a different generation time and yielding cells of different size and composition (72, 73, 86).

The final state is one of decline; the number of viable forms gradually decreases until all are dead. The death rate of the bacterial cell population may or may not be logarithmic depending on different environmental factors, such as the used growth medium and the microbe involved. Usually most of the microbial populations die in a logarithmic fashion (72, 73, 86, 89). The death curve may be complex, because after the fast reduction of the main part of the population, there can be a small population of particularly resistant cells, which can be one reason for the death curve complexities.

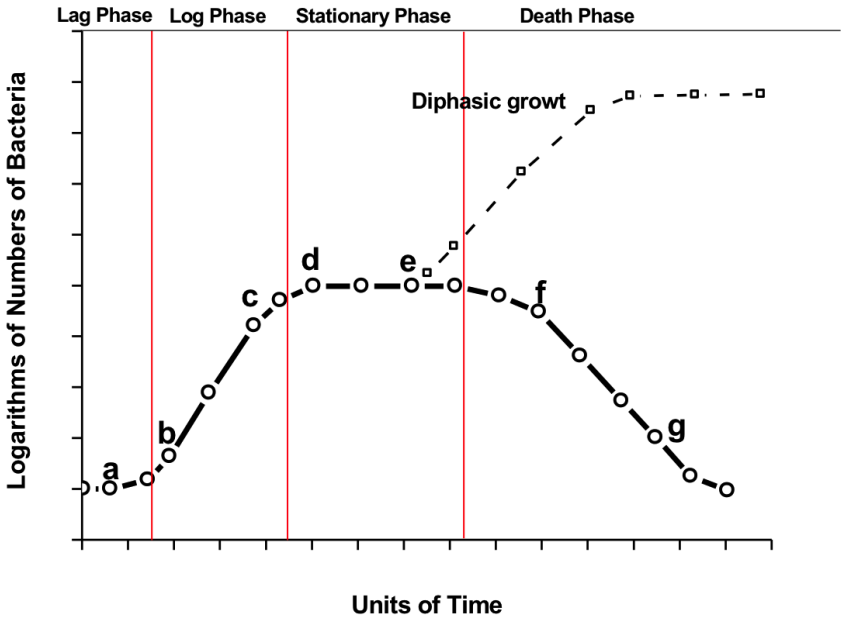


Figure 5: American microbiologist R.E. Buchanan defined a more detailed division of the growth periods in 1918 as follows.
a. *Initial Stationary Phase*. – During this phase, the number of bacteria remains constant. Plotting the results on the graph the curve gives a straight horizontal line. **a-b.** *Lag Phase* During this phase, the rate of multiplication increases with time. **b-c**. *Log Growth Phase*. – During this phase, the rate of multiplication remains constant. This means that the generation time is the same throughout. **c-d.** *Phase of Negative Growth Acceleration*. – During this phase, the rate of multiplication decreases. The average generation time increases. The organism continues to increase in number but at a slower rate than during the Log Growth Phase. **d-e.** *Maximum Stationary Phase*. – During this phase, the number of living organism remains constant, i.e., the death rate equals the rate of increase. **e-f.** *Phase of Accelerated Death*. – During this phase, the numbers fall with increasing rapidity. The average rate of death per organism increases to a maximum. **f-g.** *Logarithmic Death Phase.* – During this phase, the rate of death is constant.

2.3 Bacterial viability

We are interested in controlling bacteria and other microorganisms since their activities so directly influence our welfare. Knowledge on bacterial viability is of the essence when we attempt to provide favorable conditions for their activity and survival in the industry or as probiotics in our food. On the other hand, we need to understand bacterial cell death in trying to destroy them when they are threatening our lives, food supplies or other possessions or when we want to use them as test organisms in antimicrobial assays (72, 73, 75, 82).
The determination of the viability of the cell constantly being stressed by different environmental factors is an important part of microbiology and immunology. It is also very controversial, since a bacterial culture consists of both living and dead bacterial cells in dynamic balance. The viability is conventionally defined as the capability of cells to proliferate in sufficient medium or form plagues or colonies on solid agar, containing appropriate nutrition and physical conditions.
The colony counting assay traditionally estimates bacterial viability and killing, and a number of methods have been employed for the propagation of cultures from single cells. Most of these are laborious and time-consuming and exclude the kinetical real-time assessment because of the long incubation period. It must be emphasized here that viability and culturability are not necessarily synonyms, but of course most certainly the dead or dying bacterial cells have lost their culturability,

irreversibly. A plate counting assay does not indicate the viability of the individual cells in a population, but it merely shows that in this population there were cells capable of division and growth (86, 90-98).
The definition of viability is actually quite imprecise and full of controversy for several reasons. Though most bacteria are able to multiply in nature under a large variety of conditions, a detailed study of their activity is only possible by the examination of the growth characteristics under artificial (laboratory) conditions, and a wide range of bacterial species does not grow in these *in vitro* circumstances (99, 100) (II). A species is maintained in the laboratory by storing the cells in the deep freeze or under conditions the most suitable for their survival and by transferring to fresh media when necessary. Under natural conditions a species maintains itself by continuous multiplication, a balance being established between the rates of death and multiplication. This balance can be easily upset by stress, by changes in temperature, pH, osmolality, pressure, moisture, availability of oxygen and nutrients or by the addition of inhibitory agents (98, 98-102) (II).
This agent may be competition with other species and other factors encountered in the environments, or it can be a toxic agent such as antibiotic or some other toxin or dilution. The population balance is highly dynamic, shifting with changing conditions, but the species survives owing to the existence of the various cycles of the elements maintained in the mixed populations of micro and macro forms of life. Constituents of any one cell in time serve as nutrients for other members of the same or of different species (72, 73, 86).

Like the definition of viability, the definition of dead bacteria is also very multifaceted (86, 103-105). The absence of growth and metabolic activity in bacterial cultures or subcultures has traditionally been employed as the criterion of bacterial cell death and the result of bactericidal activity. This was discovered after German physician Robert Heinrich Herman Koch's development of pure-culture techniques in 1881. Controversially we know that viable bacteria can be dormant and unculturable, that is, they are unable to grow in the given circumstances but are not dead. Moreover, dead bacteria can generate signs of metabolic activity (86, 103-105) and produce secondary metabolites inactively by diffusing the dissolved compounds into the surroundings (105-109)**.** The decomposition of the cell population, the decrease of the cell integrity is also considered one clear indication of the cell death. However, a dying bacterial cell can maintain its bacterial integrity for an undefined time period, depending on the different environmental factors, and the cell lysis is not always a part of bacterial cell death (86, 103, 105, 109)**.** In some circumstances, viable and culturable species can alter to a viable but non-culturable (VBNC) state. These bacteria are still metabolically active and very much alive but have ceased to proliferate, however, metabolic activity is detected to change, concerning the decrease in cell morphology (alteration in the cell shape and the reduction in cell size) (74, 77, 110), in cell respiration, in nutrient transport and in the synthesis of macromolecules. This usually occurs when the environmental factors are not optimal for growth, including external stress factors such as heat, dryness, osmotic pressure, etc., much the same than in the case of spore formation in some bacterial species (90) usually to ensure the long-term survival in altered situations. This phenomenon related to halted and suspended growth is that of dormancy, which can be determined as a state of reversible metabolic stagnation. In this state, bacteria are neither simply alive nor are they dead, and they are undetectable in conventional viability assessments. In addition to the VBNC state and sporulation, bacteria dormancy can also be the result of cell injury resulting from extra- or intracellular stress (111-113). Extracellular factors are the same physical and chemical factors listed earlier, but the stress factors can also emerge from inside. This phenomenon is also called the natural aging of the bacteria and is the consequence of internal production of reactive oxygen species (ROS) (114), causing the reduction in culturability, especially in starvation induced growth arrest (83, 115-118). To conclude, bacterial cells can actually be classified in three different subpopulations according to their vital state – viable, dormant and dead cells.

These phenomena are not to be considered just random occurrences of cytological conditions, but they are examples of programmed cellular mechanisms developed to survive in conditions not optimal for cell proliferation (103, 105, 119, 120)**.**
Bacterial cells can remain in the state of dormancy, due to adaptive strategy, for some periods of time, and when spores are concerned, this period can be considerably long. Eventually, cells are likely to resume a new growth or they will enter into the death phase. If favourable environmental conditions are restored, viability is revived and cells are resuscitated concerning both the VBNC and injured bacterial cells (103, 108, 111, 113, 121, 122).

2.4 Bacterial death

The death of bacterial cells can be induced by a wide variety of chemical, physical and biological agents (38, 83, 100, 123) (II). No single group of agents can be spoken of as disinfectants. Most chemicals in an appropriate concentration induce the death of bacteria; relatively few do so in a short period of time and in a concentration that would be practical for ordinary use (72, 73). Agents that extract a bactericidal effect in certain concentrations are generally inhibitory in lower concentrations and may stimulate growth as the concentration of the agent is further reduced. All degrees of intergradation may be observed, depending on the nature and concentration of both the organism and the chemical agent as well as on environmental factors. To conclude, every substance, which in a definite concentration will kill bacteria, inhibits development in lower concentrations, and in still lower concentrations may act as a stimulant. (German physician and Bacteriologist Ferdinand Adolph Theophil Hueppe recognized this in 1896) (82, 85). This behavior is visualized in Figure 6 in the form of a disinfectant spectrum.
The antimicrobial effects are also described in detail in Figure 6, the disconnected lines indicating that no sharp line of demarcation can always be established. Furthermore, the width of the different bands for any agent varies according to the nature of the organism and the conditions of application.
The disinfectant spectrum indicates that any substances can, in certain concentrations, exert a killing effect on bacteria. In popular usage, however, a disinfectant is considered a substance that exerts a marked bactericidal effect in a relatively low concentration. In a more strict sense, the various terms commonly employed for agents intended for the control of destructive microbe action, and hence of the microorganisms themselves, can be defined as follows (44, 72, 73, 124-126)**.** The term "*sterilization*" implies the killing or the removal of all forms of life (123, 127-133).
The terms "*disinfection"* and "*disinfectant"* are used to represent the killing process (bactericidal action) induced by any agent (disinfectant) *in vitro* when employed in a suitable concentration or intensity against bacteria or their spores, whether the organism is infectious or non-infectious in character. The definitions can never be absolute in character, since it is theoretically possible that one agent might be employed in a definite concentration as a disinfectant, in a lower concentration as an antiseptic, and in a lower still concentration as a chemotherapeutical agent (72, 73).
The course on disinfection is considered an action whereby bacteria are killed in a reasonable period of time. This period is arbitrarily set in many tests but can be varied to suit the particular study or task. In Figure 6, increases and decreases in the number of bacteria with time are plotted for cultures in the logarithmic phase of growth to which different amounts of a chemical agent were added at zero time. In the presence on sufficiently small amounts of the agent, the growth curve follows the normal pattern.

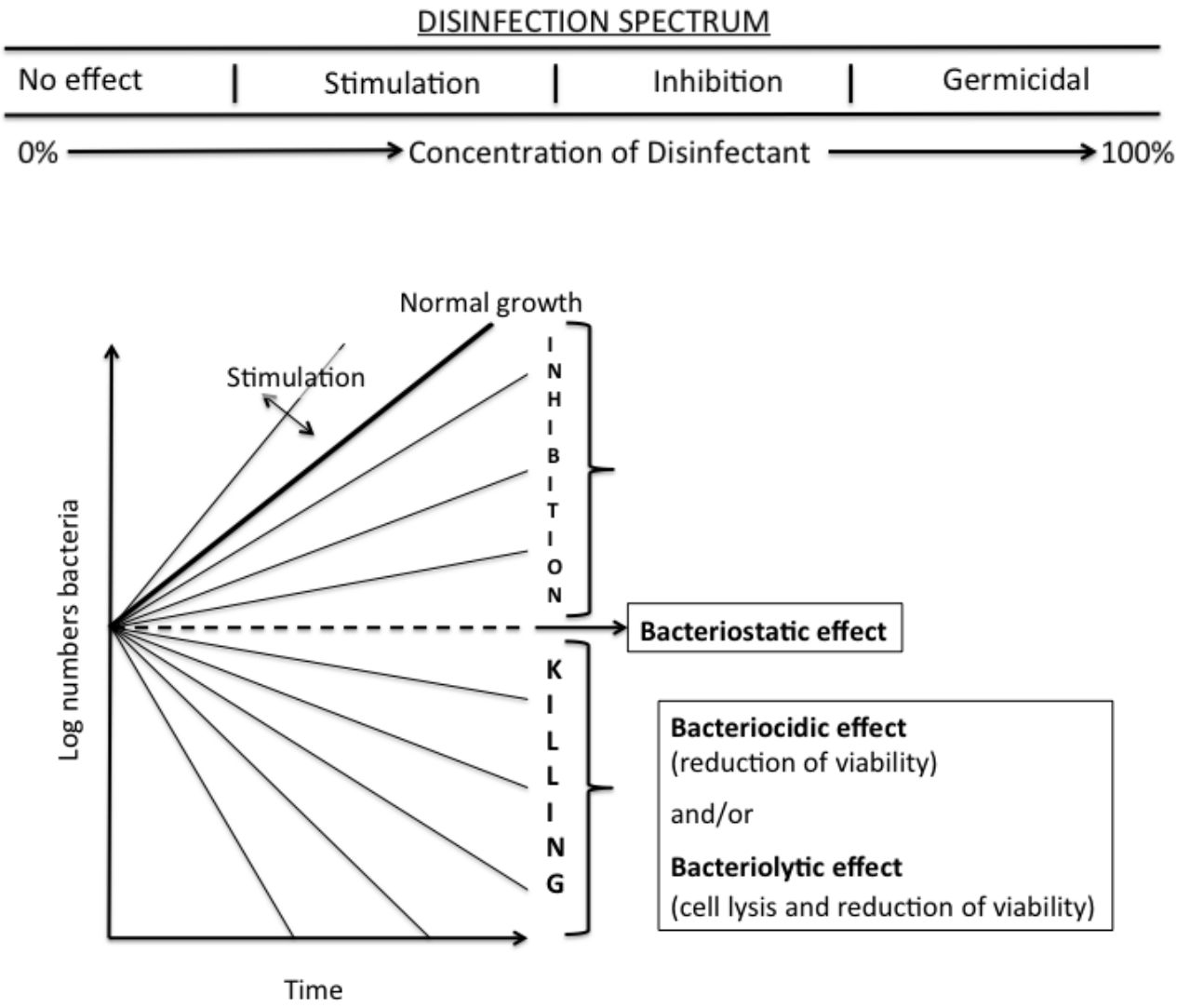


Figure 6: The infection spectrum is presented above and beneath is the simplified scheme of antibacterial effects and influence of the concentration of a chemical agent on the growth and death of bacteria (72, 73, 79, 134).

As the concentration of the test material is increased, a point is reached at which slight stimulation of growth might be noted. At still higher concentrations the rate of growth decreases below that noted in the control (normal growth in Figure 6) and, with increasing concentration, approaches zero, i.e. completes inhibition but no death of the organism in relatively short periods of time. Further increases in the concentration of the chemical results in the death of some cells per unit of time, eventually a concentration being reached at which all the organisms are killed (72, 73).
The time course of disinfection is ordinarily determined by removing measured portions of bacterial suspension containing the toxic agent at regular intervals of time, test conditions being maintained as uniform as possible. When the suspension of organism is exposed to the disinfectant under uniform conditions, not all the cells die at once. Death of bacteria in most cases is a gradual process of measurable velocity. The equilibrium between newly formed organisms and dying cells, which was noted in the preceding phase, may last for about an hour or be prolonged for several days. Once the equilibrium has been disrupted and the cells start to die more rapidly than new ones are generated, the number of viable bacteria will begin to decrease, and the stationary phase will pass gradually into a period of decline (Figure 5, 6 and 7). This interval of slow decline may represent a short lag or adjustment to the unfavourable environment in the medium. The process leading to death may be

reversible by the cell after the removal or inactivation of the cause during the first state of the process. Gradually the process becomes irreversible by the cell, but it may still be subjected to an outside factor removing the disinfectant (Figure 8). After a time, it reaches an irreversible stage, i.e. the cell is unable to multiply under favourable conditions although it may still possess other characteristics associated with living matter (72, 73).

Like the lag phase and the negative acceleration phase, this period of cell number decline is subjected to considerable variability, depending on the particular organism and the environment. Thus an accurate mathematical analysis is difficult to formulate (72, 79). The rate at which bacteria die when subjected to unfavourable environmental conditions or antimicrobial agents seems to follow a gradual and a rather orderly course throughout most of the phase, with some tendency to slow up during the later stages, as shown in Figure 5. The proper interpretation of this phase is of importance, since it is on the rate of death of bacteria that the dynamics of sterilization and disinfection are based. The decrease in number of viable organisms with time under relatively mild conditions occurs gradually and generally uniformly, i.e. the number dying during any period of time being a certain fraction of the living at the beginning of the test period (72, 73, 79).

In the study of bacterial death rates, the theory of logarithmic death has been formulated to explain the general curve obtained by plotting the number of surviving cells against time (135-137). When studying the action of heat, chemical disinfectants, and other unfavourable influences on the death of bacteria, when plotted in logarithmic form, the curve shows a close agreement to the curve for the increase in a bacterial population during its period of rapid multiplication. In the log phase, the logarithms of the number of new cells formed from a single initial cell in a given time are proportional to the lapse of time; in the logarithmic death phase, the logarithms of the proportion of the cells present that die in a given interval of time are proportional to the length of that interval. In other words, the increase and decrease alike bear a direct relation to the number of cells present at the beginning of a unit period and a logarithmic relation to any time period of greater duration (67, 135-137)**.** When the logarithms of the number of surviving bacteria after various lengths of time are plotted against time, the points will be found to align in a straight line (Figure 5 and 7).

The distribution curve (Figure 5 and 7) indicates that a constant percentage of cells are killed per unit time, which means that a straight line relationship exists when the logarithms of the number of survivors are plotted against time. The ideal relationship is indicated in Figure 6; actually, deviations from the logarithmic order of death are frequently noted. Typical deviation observed in the studies of bacterial killing include the increase or decrease of the death rate with time, the former often resulting in a marked lag period in death followed by an exponential phase (72).

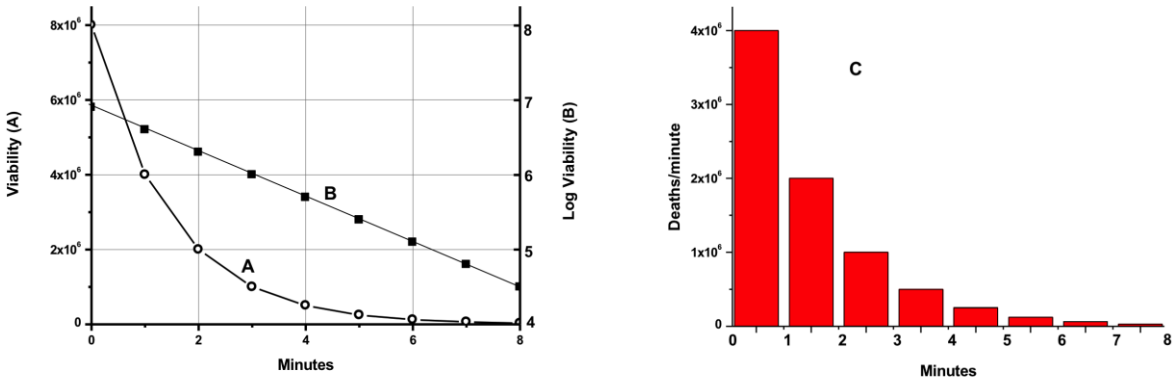


Figure 7: Plots of (A) the numbers of survivors, (B) logarithms of survivors, and (C) numbers of cells killed per unit of time against time (72, 73, 79).

We know that bacteria of different ages, or cells from cultures of different ages, differ in their susceptibility to the given agent. It has been reported that young cells are more readily destroyed than older ones (82, 135-138). The logarithmic dying pattern of bacterial cell populations can also be explained by this finding. The youngest population in a patch culture is the largest one whereas in the logarithmic dead phase the first dying population is the largest (Figure 7). This phenomenon can be dictated by the cytological resiliency of mature cells, forming more effective resistance than younger cell populations. The degradation of a particular population, proceeding in an orderly manner also brings up the question of programmed cell death (PCD), i.e. bacterial apoptosis (135-137, 139-141), although apoptosis has conventionally been outlined as the characteristics of only the eukaryote cell. PCD refers to any given form of cell death that is commenced by the intracellular death program, irrespective of the triggering factor. Apoptosis is defined as a reaction pathway, which makes the self-destruction of damaged or excessively growing cells possible on the animals. PCD has also been observed with bacteria. It has been thought that PCD has been formed generally as population response and control of the environmental stress factors. Triggering factors are partly the same as in the case of sporulation and dormancy, like disinfectants, heat, pressure, osmotic stress, DNA damages, oxidative stress, phage infection and the evasion of immune systems like complement and lysozyme (142) (I, II). The same stimuli also cause PCD on yeasts and mammals. For bacteria, as a unicellular organism, PCD is harmful for individual cells, but the advantage appears through the whole population, and in this sense the bacterial cells and the population act like a multicellular organism.

The most examined PCD mechanism of the bacteria is a so-called addiction module assisted cell death in *E.coli*, which is also called a toxin-antitoxin-system (TA). Here the gene pair produces two components: stable toxin and unstable antitoxin, preventing the lethal operation of the actual toxin. These genes were first found in low copy number plasmids of the *E.coli* where they are responsible for the post-segregational effect. When a bacterium loses this plasmid, they will selectively die. This is due to the fact that the unstable antitoxin breaks faster than the more stable toxin. In *E. coli*, the mazEF TA-system is known to activate in starvation, and the PCD population is thought to provide nourishment for the surviving cells. MazEF is also known to activate in the case of phage infected *E.coli* cells, and PCD is believed to prevent the spreading of the phage infection throughout the whole population (139).

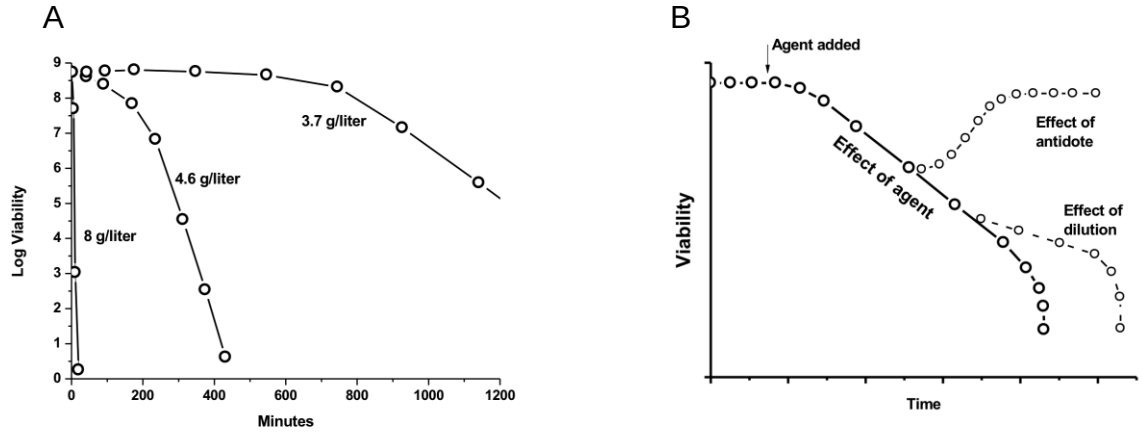


Figure 8: (A) The representation of the effect of a toxic agent on a bacterium. (B) Plots of logarithms of viable *Escherichia coli* against time in the presence of different concentrations of phenol (Redrawn from Jordan and Jacobs, Journal of Hygiene, 1944).

Disinfection studies are generally carried out under relatively drastic conditions with regard to the concentration or intensity of the lethal agent, and death follows in relatively short periods of exposure (I, II). Minor differences in the resistance of individual cells are not detected. As the concentration of the lethal agent is decreased under carefully controlled conditions, and hence the time required for disinfection to proceed to a definite end point is increased, departures from the logarithmic relationship become more pronounced (I). Jordan and Jacobs determined this behavior in 1944 (Figure 8), and it is a cornerstone of modern antimicrobial assessment as well as the basis for the antimicrobial activity assessment used in the research presented in this book. The behavior is illustrated in their study where a viable population of *E.coli* was maintained at a constant level in a control flask by the frequent addition of nutrients. Changes in the viability were determined in other cultures receiving the same treatment but to which different amounts of phenol had been added (Figure 8A). It is evident that, under carefully controlled conditions, marked deviations from the logarithmic order of death can be noted as the concentration of the toxic agent is decreased. The test reflects the balance between the life and death of bacteria in the presence of a toxic agent in an environment otherwise conductive to multiplication.

The sensitivity varies markedly among cells in any population, and this also causes bacteriostatic and bactericidal effects. It is suggested that the vital activity of the cell might be reduced with time (Figure 8A and B) of exposure to an inhibitory agent to such a low level that the cell can not recover and thus the inhibition of bacteriostasis reaching an irreversible level (108, 143). At any intermediate time of exposure the inhibition of vital activity would not be sufficient to cause damage to the cell, and it could recover to normal activity and multiply following removal or neutralization of the lethal agent. When toxin is reduced by dilution, the cell can recover normal activity if dilution is carried out to a sufficient extent; otherwise the time required for inhibition to reach the irreversible state is increased. When cells differ in sensitivity (Figure 9), similar behavior is noted with the exception that the period of time during which inhibitory effects are reversible increases. A cell sensitive to one substance can be highly resistant to another, while another cell could exhibit a reverse response. The length of the inhibitory period for the given degree of sensitivity would be controlled by the concentration of the inhibitory agent. The various observations on the disinfection process point to three factors; the sensitivity of the cell, concentration (or intensity) of the inhibitory agent, and time must be considered in any application of these processes. Accurate information on the former, the sensitivity of the cell, is always hard to define, but it can be untangled by thoroughly investigating the physical and chemical factors of experimental settings. The latter two factors are easier to determine: The agent in question is controlled by the rate at which it comes into contact with some vital component(s) of the cell and the resultant rate(s) at which dependent activities essential to multiplication are decreased. There are three major reactive systems in the cell – membrane, enzymes, and the nucleus – whose normal functioning is essential to life and multiplication (72, 73, 82).

The cell membrane can be attacked by some agents resulting in increased permeability of the cell (144). This can lead to the diffusion of essential metabolites out of the cell with resultant injury and eventual dead. A change might be induced, which could result in decreased permeability, particularly to an essential ingredient of the medium, and death. When the toxic agent reacts only with intracellular components, particularly enzymes, the rate of death of the cells will depend to some extent on the rate at which the substance can penetrate the cell membrane and diffuse through the cytoplasm (145, 146).

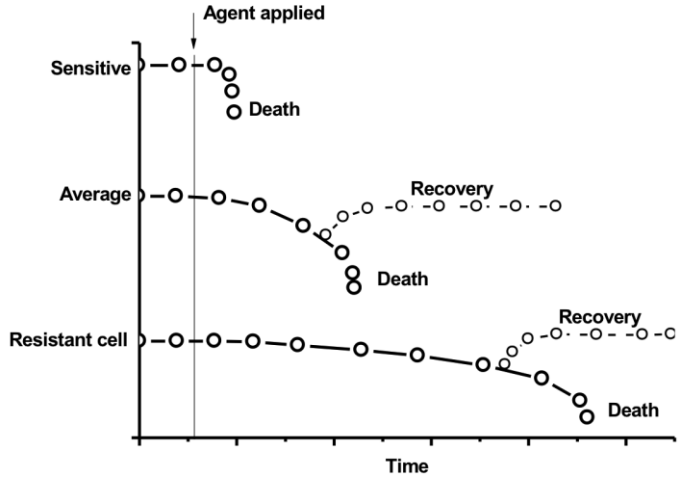


Figure 9: Schematic representation of the effects of a toxic agent on bacterial cells which vary in susceptibility.

Entry into the cell often depends on the state of ionization of the agent and/or membrane constituents; hence, pH can exert a marked influence on the rate of death. Furthermore, pH can influence the strength of the bond between the agent and cellular constituents in many instances, the firmness of union between a positively charged toxic molecule and a protein increasing within pH, and vice versa. Reaction of the agent with cytoplasmic constituents, generally proteinaceous and enzymatic in character, can be relatively specific or nonspecific. Disruption of the forces that maintain proteins in an organized state can be induced by an appropriate concentration of many agents with a widely different nature, and this denaturation may or may not be reversible. The inhibitory agent may be more specific in its action, like antibiotics, affecting specific targets (2, 12, 72, 145, 146). When the primary reaction induced by the agent results in the inhibition, neutralization, or destruction of a system involved in a reaction, or reaction chain, essential to the life of the cell and one which cannot be bypassed, death will follow as cellular activities diverge further and further from their normal dynamic balance or steady-state conditions. In some instances, the toxic agent may react with nuclear components and any physical or chemical change, resulting in the failure of the genetic material to be duplicated, which will lead to eventual bactericidal action, i.e. the inability of the cell to multiply under test conditions.

2.4.1 Chemical and biological antimicrobial and cytotoxic agents

This section mainly describes the effects of cytotoxic agents on bacterial cells, but most of them are also applicable to eukaryotes. Considerable effort has been made in understanding the mechanisms of antibacterial activation and the action of antibiotics and other biocides (2, 12, 72, 123, 145, 146). The latter refers to an agent that is unspecific and quite rapidly kills or inhibits the growth of the target cells. Antibiotics, on the other hand, have a narrower spectrum of target cells and generally one target mechanism while biocides may have multiple targets and a broader spectrum of mechanisms (2, 12, 72, 145, 146).

At a sufficiently high concentration all chemicals, including nutrients such as oxygen and fatty acids, are bactericidal and can thus be defined as biocides, though the term is restricted to substances that are rapidly bactericidal at moderate concentrations. In contrast to lethal radiations (which damage DNA) and most bactericidal chemotherapeutic agents (which interact irreversibly with various active metabolic systems), most biocides act either by dissolving lipids from the cell membrane (detergents, lipid solvent) or by damaging proteins (denaturants, oxidants, alkylating agents, and sulfhydryl reagents).
A description of all chemicals possessing bactericidal properties is not feasible, but a consideration of the different classes into which these agents have been grouped is both useful and instructive. There is no uniform law governing their action; many variable factors are involved, and each may modify the efficiency of the destructive process. The rate of killing by biocides increases with concentration and temperature. Anionic compounds are more active at low pH and cationic compounds at high pH. This effect results from the greater penetration of the undissociated form of inhibitor and possibly also from the increase in opposite charge in cell constituents (72, 73).
The effectiveness of the antimicrobial and chemotherapeutic agent against the microbe can be estimated from the minimal inhibitory concentration (MIC), which is the lowest concentration of the agent that inhibits the growth of the target microbe. The lowest concentration causing the target cell to die is the minimal lethal concentration (MLC). The dose or concentration required to kill 50 % of the tested population during the test period is referred to as the lethal dose (LD_{50}) and lethal concentration (LC_{50}) respectively (72, 73).

Distilled water: There is no generally accepted view about the antimicrobial impact of distilled water. Osmotic stress is known to destroy both eukaryotic and prokaryotic cells to some extent, and different bacterial species are known to be variably susceptible to distilled water (72, 73).

Salts: Pickling in brine, or treatment with solid NaCl, has been used for many centuries as a means of preserving perishable meat and fish. Bacteria vary widely in susceptibility. Though physiological saline (0.9 %, 154 mM, NaCl) is widely used as a diluent for bacteria, it is not very suitable; a balanced salt solution, containing Mg^{++} and buffer, permits much better survival. Strains vary widely in their ability to survive in distilled water; some of the lethal actions, however, are due to traces of heavy metal ions, which are more bactericidal in the absence of competitive ions (72, 73).

Metals: The various metallic ions can be arranged in a series of decreasing cytotoxic activity with quicksilver (HgII), lead (PbII), cadmium (CdII), Zinc (ZnII) and silver (AgII), at the head of the list, as they can be effective at the part per million (ppm) levels. The concentration required for killing is markedly affected by inoculum size (79, 82, 147).
Sulfhydryl compounds can readily reverse the antibacterial action of Hg^{++}. Similar inhibition can be obtained with those enzymes whose activity involves an SH- group.
Before penicillin, compounds including mercury and silver were used as disinfectants and antiseptics. The organic compounds of arsenic, bismuth and antimony have been used in the chemotherapy of syphilis. Copper salts have had a great importance in agriculture.
Forming very strong bonds with functional groups of proteins, like the imidazolium nitrogens of histidine, thiolates and cysteine residues, the metal ions can frequently inhibit enzyme or some other biological activity of the molecule (147).

Titanium dioxide (TiO_2) is a strong oxidizing agent widely used in nanoparticle form as a white pigment in paints, in toothpastes and in pastry products. TiO_2 nanoparticles are also used as a coating on microbe free surfaces and in microbe free plastics. It has also been applied in cleaning and neutralizing mold-damaged buildings. The use of TiO_2 is controversial, since some studies claim

high toxicity and others a total non-toxicity of the product. When exposed with UV light (photo catalytic), it reacts and breaks organic compounds causing a cytotoxic effect to both prokaryotes and eukaryotic cells (148-153).

Inorganic anions: Inorganic anions are much less toxic than some of the cations. Boric acid has found wide use as an antiseptic agent (72, 73, 79, 82).

Halogens: Iodine binds irreversibly to proteins (e.g. by iodinating tyrosine residues), and it is an oxidant. The tincture of iodine (a 2 to 7 % solution of I_2 in aqueous alcohol solution containing KI) is a rapidly acting bactericide. It is a reliable antiseptic for the skin and for minor wounds, but it has a painful and destructive effect on exposed tissue. I_2 complexes spontaneously with detergents to form iodophors, which provide a readily available reservoir of bound I_2 in equilibrium (154-156).
Chlorine antiseptic was introduced in the first part of the 19^{th} century to prevent the transmission of puerperal sepsis by the physician's hand. Chlorine combines with water to form hypochlorous acid (HOCl), a strong oxidizing agent (38, 157-159):

$$Cl_2 + H_2O \rightarrow HCl + HOCl$$

or

$$Cl_2 + 2\,NaOH \rightarrow NaCl + NaOCl + H_2O$$

Hypochloride solutions are used to sanitize clean surfaces (200 ppm Cl_2) in food and dairy industries and restaurants. Cl_2 gas, at 1 to 3 ppm, is used to disinfect water supplies and swimming pools, and in hospitals 3000 ppm or higher chlorine solutions are used, as they are effective against *Clodstridium difficile* spores (160, 161).
Chlorine is a reliable, rapidly acting disinfectant for such "clean" materials, but it is less satisfactory for materials containing organic matter that can react rapidly with the Cl_2.

Other oxidants: Hydrogen peroxide (H_2O_2), in a 3 % solution, was widely used as an antiseptic. Bacteria vary in their susceptibility, since some species possess catalase (162, 163). Potassium permanganate ($KMnO_4$) is used as an antiseptic in 1/1000 concentrations (164).

Peracetic acid: (CH3CO-O-OH), a strong oxidizing agent is used as a vapor for the sterilization of chambers for germ-free animals (165, 166). These oxidants, as well as halogens, presumably act by oxidizing SH and S-S groups of enzymes and membrane components, which are also referred to as the bacterial safe groups (79, 82, 167)

Alkylating agents: Formaldehyde and ethylene oxide replace the labile H atoms on $–HN_2$ and –OH groups, which are abundant in proteins and nucleic acids, and on –COOH and –SH groups of proteins. These alkylating agents, in contrast to other disinfectants, are nearly as active against spores as against vegetative bacterial cells, presumably because they can penetrate easily (being small and uncharged) and do not require H_2O for their action (72).

Surface-active agents: These compounds (surfactants) are generally called synthetic detergents. Such compounds, like fatty acids (soaps), contain both a hydrophobic and a hydrophilic portion; they therefore form micelles (large aggregates) in aqueous solution in which only the hydrophobic portion is in contact with the water. They can similarly form a layer that coats and solubilizes hydrophobic molecules or structures. Anionic detergents are only weakly bactericidal, perhaps because they are

repelled by the net negative charge of the bacterial surface. Non-ionic detergents are not bactericidal and may even serve as nutrients (72, 167).

Cationic detergents: Cationic detergents are active against all kinds of microbial cells. The most effective types are the quaternary compounds, containing three short-chain alkyl groups as well as a long-chain alkyl group. These compounds are used for skin antisepsis and for sanitizing food utensils. They act by disrupting the cell membrane, causing the release of metabolites; in addition, their detergent action provides the advantage of dissolving lipid films that may protect bacteria, and they leave a tenacious bactericidal surface film on the treated object (72, 167).
Polyhexamethylene biguanide **(**PHMG) is a biocide whose effect is based on its strong cationic reservation in a physiological pH area. The positively charged PHMG molecule attaches to the negatively charged membrane of the cell. PHMG prevents the cell membrane's function by disrupting nutrient (especially sugar molecules) intake. Bacterial cell are more susceptible to PHMG than molds (fungi), which can still survive in 50 to 200 ppm concentrations. It has been used as a biocide and in the disinfection of industrial instrument in 200 to 5000 ppm concentrations (168-171). Because mammalian cells (especially human lung cells) are more susceptible than microbes, the usage of PHMG was banned in all applications by the European Union beginning from 1.2.2013 [(EC) 1849/2006, 2011/391/EU and 2012/78/EU].

Acids and alkalis: Strong acid and alkaline solutions are actively bactericidal (72, 167)**.** However, mycobacteria are relatively resistant; it used to be a common practice to liquify the sputum sample of patients by the exposure of 30 minutes to 1 N NaOH or H_2SO_4. This procedure depends on the survival of a fraction of populations rather than the complete resistance of the bacteria. Gram positive (gram (+)) *staphylococci* and *streptococci* also frequently survived (82).
Weak organic acids exert a greater effect than can be accounted for by their pH; the presence of highly permeable undissociated molecules promotes the penetration of the acids into the cells. The increasing activity with chain length suggests a direct action of the organic compound itself. Lactic acid is a natural preservative in many fermentation products; the salts of propionic acid are now frequently added to bread and other foods to retard mold growth (81).
Acetic acid is an effective antibacterial compound, and it is also used for the preservation of food. Acetic acid has been known to prevent the growth of organisms, otherwise naturally resistant to many antimicrobial compounds, such as gram (-) *Pseudomonas aeruginosa,* at concentrations lower than 1 % (172). Acetic acid is known to act as a mild antibacterial and antifungal agent in the vaginal lubricants of humans and other primates (173).

Alcohols: The antimicrobial action of the aliphatic alcohols increases with length up to 8 to 10 carbon atoms, above which water solubility becomes too low. Ethanol is an antimicrobial agent killing organisms by denaturing their proteins and dissolving their lipids, being effective against most bacteria and fungi (100, 123). The disinfectant action of alcohols, like their denaturing effect on proteins, involves the participation of water. Ethanol is the most effective in 50–70 % aqueous solution; at 100 % it is a poor disinfectant, and *Bacillus anthracis* spores have been reported to survive for as long as 50 days. The bactericidal activity of ethanol is negligible at concentrations below 10 to 20 %. Although ethanol has received the widest use, the common laboratory solvent methanol is also cytotoxic, utilizing similar mechanisms to ethanol. Isopropyl alcohol is less volatile and slightly more potent than methanol and ethanol. Some organic disinfectants like formaldehyde and phenol are less effective dissolved in alcohols than in water because of the lowered affinity of the disinfectant for bacteria relative to the solvent.

Phenols: Phenol is both an effective denaturant of proteins and a detergent. Its bactericidal action involves cell lysis. In sufficient concentrations, phenol is effective against vegetative cells of

microorganisms. Most bacteria are killed within 5 to 10 minutes by a dilution of 1 % at room temperature. Bacterial spores are more resistant and the spores of *B. anthracis* may survive in 5 % phenol for 24 hours or longer (123, 167). The antibacterial activity of phenol is increased by alkyl or halogen (like chlorine) substituent to the ring, which increase the polarity of the phenolic OH- group and make the rest of the molecule more hydrophobic; the molecule becomes more surface-active and its antibacterial potency may be increased a hundredfold or more.

Other organic solvents: Organic solvents such as ether, benzene, acetone, chloroform or dimethyl sulfoxide also kill bacteria but are not reliable disinfectants. However, the addition of a few drops of toluene or chloroform to saturate aqueous solutions will prevent the growth of fungi and bacteria. Glyserol is bacteriostatic at concentrations exceeding 50 % and is used as a preservative for vaccines and other biological products (81, 123).

Antibiotics: Chemotherapeutic agents are defined as chemicals that can interfere directly and selectively with the proliferation of microorganisms. Accordingly, their essential feature is selective toxicity. Some of these drugs are bacteriostatic, the inhibition of growth being reversed when the drugs are removed. Others are bactericidal, exerting an irreversible, lethal effect (72, 146, 174, 175).
Antibacterial chemotherapy launched in Germany in 1935, when Domagk developed a dye, Prontosil, which cured streptococcal infections. The dye proved inactive *in vitro*. A year later in France, Tréfouel excreted a simpler, colourless product, sulphanilamide, which was active *in vitro*. This compound was then used directly as a drug and was soon succeeded by more potent derivates; the class is known in medicine as the sulphonamides, a competitive inhibitor of folate synthesis (Figure 10). Since then thousands of antimicrobial compounds have been discovered by screening for activity *in vitro* (72, 82, 85, 174).
The success of sulphonamides renewed interest in antibiotics, which are defined as antimicrobial agents of microbial origin. Pasteur, who observed the sterilization of anthrax bacilli in a contaminated culture, first recorded inhibition of some microbes by others in 1877. In the next half century, a number of similar accidental observations were reported.
Fleming reported in 1929 that a contaminating colony of the mold *Penicillium notatum* lysed the adjacent colonies of staphylococci. Ten years later Chain in Oxford showed that, after purification the active material, the β-lactam antibiotic called penicillin (Figure 10) was stable and it proved effective for certain infections. The success of penicillin encouraged the systematic search for additional antibiotics.
The term chemotherapeutic is sometimes used to distinguish synthetic compounds from antibiotics, but Ehlrich's original definition is more useful: those antimicrobial compounds that are nontoxic enough to be useful in therapy. Some drugs discovered as antibiotics are now produced by chemical synthesis (100, 146, 176) (II). The difference between an irreversible, bactericidal action and a reversible, static action does not simply depend on whether the agent reacts reversibly or irreversibly with its receptor, but on whether an irreplaceable element of the cell is irreversibly damaged. Three general bactericidal actions can be recognized: 1) DNA or RNA 2) cell membrane 3) any enzyme or protein required for protein synthesis (Figure 10) (12, 67, 146, 176-180).

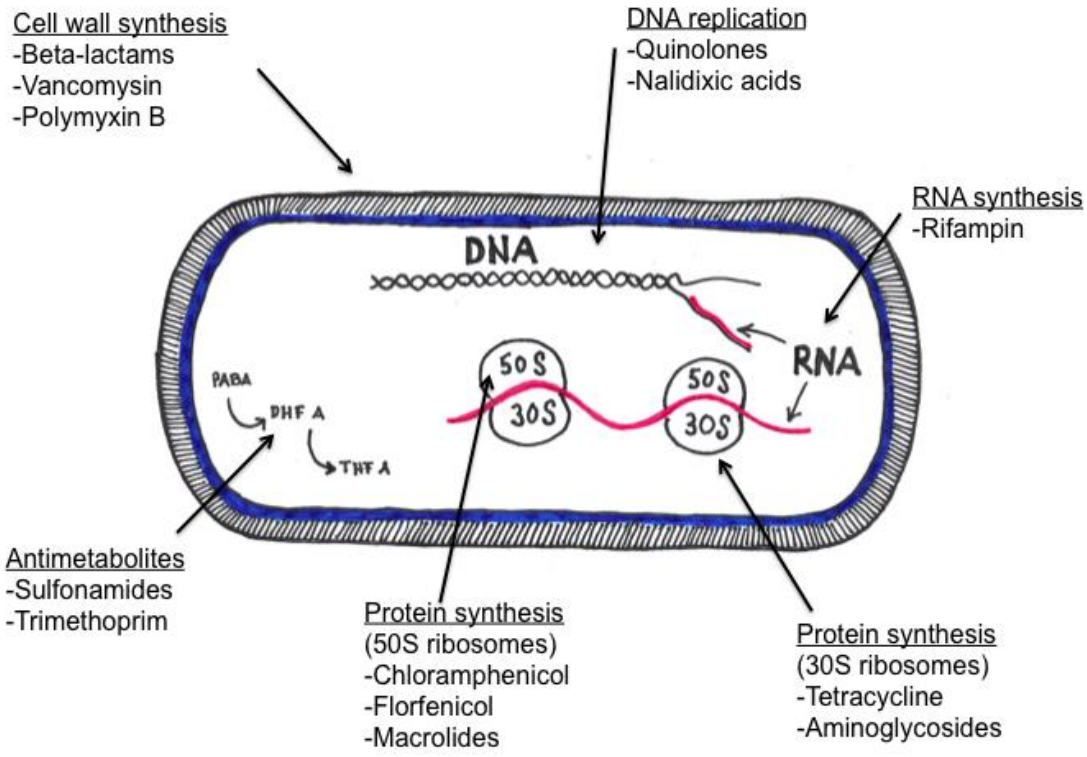


Figure 10: The targets of different classes of antibiotics in the bacterial cell. 50S and 30S refer to the prokaryotic ribosome subunits. Sulfonamides are competitive inhibitors of the folate (folic acid) synthesis in bacteria PABA (para-aminobenzoic acid), DHF A (dehydrofollic acis) and THF A (tetrahydrofollic acis) (72, 146, 175).

Mycotoxins: The (181-183) group of various cytotoxical agents produced by fungi is called mycotoxins. The spectrum of these secondary metabolites of the fungal cells is very broad, affecting both eukaryotic and bacterial cells. Very often these products are antibiotics, like antibiotic penicillin derived from *penicillium* fungi (72, 85, 184-188). *Fusarium* mycotoxins, like moniliformin, deoxynivalenol (vomitoxin), fumonisin, beauvercin, enniatin and trichothrcenes (T2-toxin), are cytotoxic agents produced by more than 50 species of the *Fusarium* genus (189-195). *Aflatoxins*, poisonous to both animal and bacterial cells and produced by *Aspergillus flavus* and *A. parasiticus*, are a widely studied group of mycotoxins with 14 different classified subtypes. Aflatoxins and their metabolites interfere with DNA, causing mutations and delays in cell cycle progression. In eukaryote, aflatoxins are reported to mutate the signaling of the apoptosis via gene *p53* (196-199). *Ochratoxins* (A, B and C) and Patulin are the secondary metabolic products of certain *Aspergillus* and *Penicillium* species and are also reported to be genotoxic to both eukaryotic and prokaryotic cells (200-202). *Cytochalasins* (A-G) are secondary metabolites of certain *Arpergillus* and *Helminthosporium* species. Cythochalasin binds to actin filaments and blocks polymerization and the elongation of actin. It will also stagnate muscle constriction and prevent the neutrophils to phagocytose their target by halting the ingestion requiring the actin filament activity (181-183)

Immune systems: All animals and plants have a system protecting them against invading pathogens, fusions and hypertrophy of the cells and tissues. Even bacterial cells have enzymatic activity against phages. All these systems are, at their simplest, highly complicated. The term "immune" derives from Latin *immuns*, i.e., exempt from "charges" (taxes, expenses). However, for nearly a century and a half the term "*immunity*" has denoted resistance to possible attack by infectious agents. In

biomedical research, immunology refers to the field of study of host defense mechanisms against infectious diseases and homeostasis maintenance, but it can also be expanded to the concept of waste removal, because the same systems participate in waste clearance.
The generalization is that the most developed systems with adaptive immunity are found in vertebrates, which are described in more detail in the next section. Simpler life forms possess only humoral mechanisms against invaders. Insects have already developed a cellular response (hemocytes are cytotoxic cells) working alongside the humoral system (haemolymph = insect blood), and their immune system functionally resembles the innate immune system of the vertebrates (IV). The humoral part of the insect blood, haemolymph, contains direct antimicrobial properties compared with that of a serum in vertebrates. This is not based on the complement system but on antimicrobial peptides (203), the cascades that regulate coagulation and melanization of hemolymph and the production of reactive intermediates of oxygen and nitrogen (204) (IV).
Fish have a functional complement system (205) (I). Some fish species have already developed a system producing an adaptive immune response with lymphoid tissues and B-lymphocytes producing antibodies. These immunoglobulins augment the phagocytizing cells to destroy invading pathogens (206). Amphibians, reptiles and birds have a distinguished innate and adaptive system with separate functional and structural characteristics (207).

2.5 Assesment of viability and cytotoxic effect with luminescent methods

2.5.1 Viability assessments

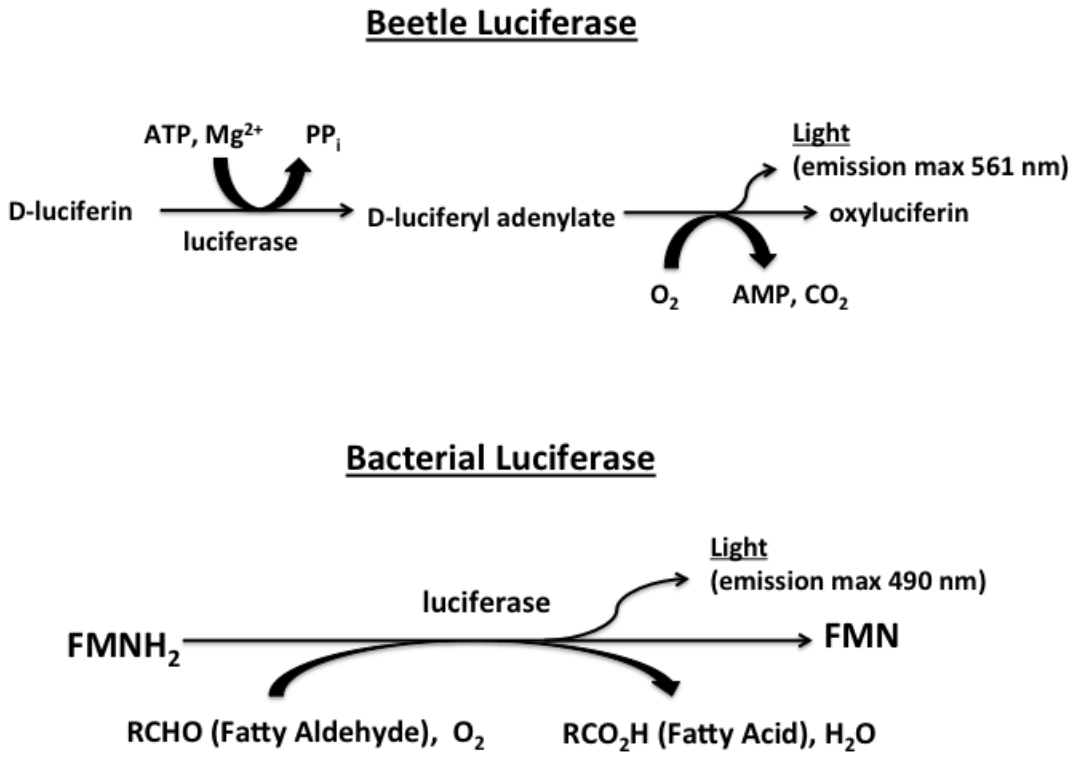
In general, we know that the definition of viability, when bacterial cells are concerned, can be very disconcerting and troublesome. The time course of the disinfection is ordinarily determined by removing measured portions of bacterial suspension containing the toxic agent at regular intervals of time and then determining the number of viable cells in the samples by dilution and plating. This conventional method can also require the neutralization of the toxic agent in subcultures to such an extent that it is no longer inhibitory to the proliferation of the viable cells (80).
The customary concept of dead bacteria is that they cannot multiply when transferred to nutrient agar or broth. As mentioned earlier, this concept can be fallacious in some instances, since “death” to some degree can result from unculturable bacterial species, dormant cells or can be influenced by the nature of agar or broth. Death can therefore be, from the conventional point of view, partially a result of the circumstances under which viability is determined, and this is why different modern techniques have been developed to assess bacterial cell viability in a more detailed manner (44) (II).
The kinetical approach by using colony counting is laborious, requiring a huge amount of plates and, moreover, the results are not obtained on a real time basis, since the plates demand a long incubation period.
OD turbidity measurement indicates the growth but does not differentiate between live and dead bacteria in the measurement solution, which restricts the application of this method (II) (208, 209); however, by using the special staining procedures, the viability analysis can be operated with spectrophotometry (78, 176) (II).
Microscopy is a widely used method for viability assessment. This is traditionally based on mobility assay or cell staining. Fluorescence microscopy provides a modern application with several fluorescent-staining protocols for viability assessment (210). Fluorescent staining methods are also applicable in flow cytometery, a widely used method for assessment (78, 211, 212).
A common disadvantage for the previously listed applications is that they require specific staining procedures and are mainly end-point methods, lacking functional real-time qualifications. This is why the approach was to design the cell producing a signal directly correlating with the viability. In

order to reliably monitor changes in bacterial viability and cell number, real time data from the whole incubation period is necessary (II). One-step or end-point measurements are usually faster and easier to repeat; however, the data from the incubation period is incomplete and the non-kinetic method only provides rough estimations concerning bacterial killing and viability. Effects of different agents and intra and extra cellular factors may alter the results greatly in different measurement environments. Alteration caused by factors like temperature, pH, incubation time, the size of inoculum and the concentration of the tested antimicrobials are conveniently visible in the real time kinetical measurements and can be revealed in the function of time (180, 213, 214).
By using reporter genes, cells can produce proteins, like luciferases and fluorescent proteins, with measurable functions directly connected to the cell viability. The kinetical real-time method, based on the bacterial cell emitting bioluminescence directly correlated with the viability of the cell, is utilized in this book and described in more detail in the next sections (78, 100, 176, 215-217) (I, V, II).

2.5.2 Bioluminescence

Bioluminescence (BL) is determined by the ability of living organisms to produce a light signal (218)**.** The phenomenon is widely spread throughout the globe, mainly among insects and bacteria but also among other species (219, 220). BL has been known since the antiquity and has also been mentioned by Aristotle (384 – 322 BC) (221). British chemist and natural scientist Robert Boyle made the first experiments with the phenomenon in 1668. However, the term "*luminescence*" occurred for the first time in 1888 when it was described by the German scientist Eilhardt Wiedemann (222).
BL producing systems can be divided into a few different categories. First are the proteins directly emitting light, with no co-factors or enzymes required, only the excitation with proper wavelength of light (223, 224). These proteins emit fluorescence, and the most applicable of these is GFP from jellyfish *Aequorea victoria*, commonly used in numerous methods assessing viability and metabolic activity. GFP genes can be transformed to other species. The methods using GFP are versatile, and after the gene from *A. Victoria* was first transferred (225) into a bacterial cell, producing an explicit fluorescent signal (223, 226), the use of GFP techniques in microbial analysis in various applications has expanded and brought new GFP variants, like the enhanced green fluorescence protein (EGFP) (227-239) (II). GFP itself is very stable, because the chromophore responsible for fluorescence emission is located inside a barrel-like structure (240). The wide range of GFP expressions among different organisms are enabled by autocatalysis or ubiquitous co-factors (223, 224). As a reporter gene, GFP works without any additional substrates or cofactors, and the fluorescent signal is linked to the number of bacterial cells. However, it cannot be used in bacterial killing assays, since GFP is stable and remains fluorescent in dead cells (99, 176). GFP is highly fluorescent and accumulates within the bacterial cell during its growth (78, 99, 100).
The second class of BL systems consists of luciferases, which are proteins acting as an enzyme, catalyzing (reduction-oxidation) reactions and producing BL as a byproduct (219, 220, 226, 241-244). There are many different kinds of emitting systems in this category. The most exploited ones in biotechnology are beetle and bacterial luciferases, but similar enzymes have been discovered from fungi and sea animals like *Renilla reniformis* and medusa *A. victoria* (245, 246). Both bacterial and beetle luciferases are widely used in multiple applications and techniques in biotechnology (99, 100, 142, 176, 216, 217, 247-249) (I, V, II, IV). The system producing light is basically the same in all BL emitting cells, consisting of luciferase enzyme and lusiferin acting as a substrate (219, 220, 244). The genes of these systems are normally found under the same operon, and they are easy to transfer to other cells and even to other species, generating a completely new BL emitting cell. Almost all of the various luciferase systems have one common factor binding their function together. A luciferase

catalyzed reaction, where lusiferins as a substrate are oxidized in the presence of molecular oxygen, produces light emission alongside other products (Figure 11). The luciferase bound intermediate is formed and degraded, electrically exited and discharged, and in the end photon is emitted. The enzymes of the various systems are not always called luciferases, but the name is used here as a generalized term, describing the ubiquitous function (219, 220, 244).
The best recognized luciferase systems are those discovered from fireflies like *Photinus pyralis* and *Pyrophorus plagiophthalamus.* These systems are a part of the beetle luciferases, and in general these enzymes catalyze the oxidative decarboxylation of benzothiazole luciferin in the presence of ATP-Mg. In the reaction the light signal, with the emission peak at 561 nm, is formed in a two-step reaction (Figure 11), as cellular energy is converted into light emission utilizing ATP, O_2 and luciferase as mono-oxygenase (219, 220, 250-259). The 62 kDa beetle luciferase is encoded by the gene *lucFF*, reported to have 45–50 % homology at the amino acid sequence between different beetle luciferases (219). Because the system is dependent on cellular ATP, the beetle luciferase system is optimal for cell viability assessments, tough the application needs an additional substrate. The biochemical pathway to the synthesis of beetle luciferin is still unknown. It is not known whether the cells contain the genes to produce the enzymes for luciferin synthesis or if the genes are gained by ingestion from other bioluminescent species, like symbiotic bioluminescent bacteria (253, 260).



11: The light emission of both beetle and bacterial luciferace systems (260, 261).

All species producing BL are gram (-) bacteria (262). The main reservoir for bacterial luciferases is found under the sea from marine bacteria, belonging to genera like *Photobacterium*, *Aliivibrio*, *Shewanella* and *Vibrio.* Most of these live in symbiosis with fish, plants or other species (245). However, bacterial luciferases are also found in terrestrial species. Bacteria belonging to genus *Photorhabdus* (previously called *Xenorhabdus*) can form highly luminescent cells. *Photorhabdus luminescens* is a pathogen living in the gut of an entomopathogenic nematode of the family Heterorhabditidae. When the insect is infected with the nematode, *P. luminescens* is released into the

blood stream and rapidly kills the insect host. The lux-gene used in the studies presented in this book originates from *P. luminescence* (263-272) (II). Other *Photorhabdus* species, like *P. asymbiotica*, have also been found to be human pathogens populating the infected wounds (272).

The bacterial luciferase catalyses the oxidation of a long chain fatty aldehyde and reduced flavin mononucleotide (FMN/$FMNH_2$), simultaneously emitting BL with the emission at a maximum of 490 nm (249, 260, 273-275) (Figure 11). Bacterial luciferase is a chimeric protein with two non-identical subunits encoded by separate genes (*luxA* and *B*) encoding α- (~40kDa) and β- (~35kDa) subunits, of which the first one, the α-subunit, includes the catalytic center (276). The yield of the photons is reported to be 0.2–0.3 per reacting $FMNH_2$, and the bacterial cell can emit 10^4–10^5 photons per second (219, 220, 244). In the *lux*-operon, these two genes are flanked by three additional genes (*lux C, D* and *E*), which are involved in the synthesis of its fatty aldehyde substrate. They encode reductase, transferase and shyntethase, respectively, i.e. the enzymes required for substrate formation, reducing long fatty acids to aldehydes.

The reaction catalyzed by bacterial luciferase is oxygen-dependent, and in normal *in vitro* circumstances, using the aerobic or facultative aerobic bacteria gain of oxygen is not the limiting factor in the light production (277). The amount of $FMNH_2$, however, is dependent on the metabolic activity of the cell, which is also the basis of its use in viability assessment, but some factors can affect the redox potential of the cell, also affecting the amount of $FMNH_2$ and the level of BL production (278). Additionally, the amount of light emitted can vary between different strains of bacteria (279).

Using luciferases as reporter genes (244) is a common method for measuring the number of bacterial cells, as the genes encode products linked to the viability of cells, for example beetle luciferase (*lucFF*) from the firefly (244) or bacterial luciferase (*luxAB*) *and (luxCDABE)* (244) from the marine bacteria *V. harveyi* and terrestrial bacteria *P. luminescens* respectively. Expressions of these genes in bacteria produce BL (280). Bacterial luciferase gene cassette *luxCDABE* also contains genes for substrate, and expression of the whole gene cluster produces a BL emitting system, lacking the need for external substrate addition (I, II, III, IV, V, VI, VIII). However, in many cases bacterial luciferase is cloned in the form of luxAB-fusion, containing only the genes for the actual luciferase structure, lacking the substrates (99, 100, 217, 281) (I). This construction also enables the use of the gene expression in other species, especially in some eukaryote cells (282). Using these reporters, bacterial viability and the effects of different antimicrobial agents, such as antiseptics, toxins, antibiotics and especially the function of immune mechanisms such as phagocytic activity and the serum complement system, can be studied (78, 99, 100, 134, 142, 176, 217, 248, 281, 283-285) (I, II, III, IV, V, VI). When *luxAB* as well as the beetle luciferase lucFF are used, the inconvenience is that luciferase systems require the addition of an external substrate (100, 281) (I). The reaction is dependent on substrate transport into the cells and is thus strongly affected by external factors such as pH. In constructs using luxAB, long chain fatty aldehydes, like decanal, are added to induce light emission.

Bacterial luciferases have been introduced to gram (+) bacterial species like *Streptococcus pneumoniae* and *Staphylococcus aureus* (249, 286) by using only the *luxAB*-construct, when the BL production level is comparable with that of gram (-) bacteria (277), or by altering the *lux* gene composition to be suitable for the gram (+) transcription system. This was done by dismantling the whole lux cassette and by adding the ribosome-binding site to every gene. Using this method, the bacterial luciferase gene *luxCDABE* from *P. luminescens* has been altered to *luxABCDE*, expressed successfully in *Streptococcus pneumonia* and *S. aureus*, resulting in a cell with high BL emission (249, 286). The latter construct works in gram (-) bacteria and is also utilized in the *E. coli* construct used in the studies presented in this book (II). LuxABCDE has been successfully transferred into the eukaryotic cell *Saccharomyces cerevisiae* by adding a yeast promoter in the lux-cassette, resulting in constitutively bioluminescent yeast cells (287). As mentioned earlier, $FMNH_2$ is the limiting factor in bacterial luciferase BL production. Especially in gram (+) species, the shortage of $FMNH_2$ is

resolved by cloning the NADPH-FMN-oxidoreductase gene (*frp*), the genuine construct from gram (-) species like *V. harveyi*, whose enzyme produces $FMNH_2$ from FMN.
Bacterial luciferases from different species have structural and functional variations. The enzyme from *P.luminescens* is classified as a "slow" decay luciferase, slow referring to slower binding of flavin and thus slower production of intermediates. Slow decay luciferases are more stable and especially more resistant to temperature alterations (288, 289). Luciferases from marine bacteria are, on the contrary, fast decay enzymes, and though the structural difference between these two types is only one amino acid residue, it has an enormous effect on the functionality. Marine bacteria exist in lower temperatures and require a high saline environment. The temperature maximum of the luciferase from *A. fisheri* is only 30 C, but luciferase from *V. harvey* has been reported to be stable still at 37 C (290). The luciferase enzyme from *P. luminescens is known* to be stable still at 42 C; this and the fact that it works in lower saline concentration makes it more applicable for *in vitro* assessments (290, 291) (II, V).
When *luxAB* genes from *V. harveyi* were transferred in *E. coli* (280, 281, 291), the problem with this construct was that during the logarithmic growth phase the BL signal elevated considerably more than was indicated by the OD signal, and at the beginning of the stationary phase, BL emission collapsed (292) (II). The same phenomenon was observed even by using *luxCDABE* from *V. fisheri* in *E. coli* and with *luxABCDE* from *P. luminescens* in *S. aureus* (293, 294). The suggested reason for this decreasing BL was suggested to be the low optimal temperature of the *V. hareveyi* luciferase and decline in the supply of the intracellular reducing power of the luciferase enzyme (293). The same phenomenon was observed even by using *luxCDABE* from *V. fisheri* in *E. coli* and with *luxABCDE* from *P. luminescens* in *S. aureus* (293, 294). When *E. coli* was transformed with the *luxABCDE* from *P. luminescens* such a decrease of the BL emission was not observed (II). On the contrary, during the logarithmic growth phase, the BL signal did not increase either in plasmid derived *E. coli* or chromosome derived *P. luminescens* systems (II). The same has been reported when *luxABCDE* genes from *P. luminescens* were integrated into the *E. coli* chromosome (295). The availability of fatty aldehyde seems to be the limiting factor in *P. luminescens* BL emission (280, 296). When a surplus of substrate, i.e. fatty aldehyde like decanal, was added to the growth medium of *E. coli* transformed with luxABCDE from *P. luminescens*, the BL signal demonstrated a remarkable increase (II).

Quorum sensing

There is a certain census conducted in a bacterial population, and this consensus of population density is referred to as quorum sensing. Many genes, operons or regulons have been found to be only expressed when the population's cell density strongly increases (297-306). The best-characterized system is the LuxR-LuxI system found in *V. fisheri* (Figure 12) (307-310). This system is based on the production of an autoinducer protein VAI-1 encoded by luxI. The inducer is diffused to intra- and extracellular surroundings. LuxR acts both as a receptor and as a VAI-1 dependent transcriptional regulator of the lux-operon, binding upstream of the *lux*-promoter. A substantial amount of the auto-inducer, i.e. high cell density, is needed for the induction of the BL system (307). High bacterial cell densities, reaching up to 10^{10}–10^{11} bacterial cells/ml, has been observed in the *V. fisheri* symbiotic light organs of some marine fish. On the other hand, the same bacteria in seawater are known to reach densities less than 100 cells/ml but not emit BL. Auto-inducers of quorum sensing bacteria are also known to send signals to heterologous bacterial species (306, 311).
Quorum sensing systems homologous to LuxR-LuxI, are also known to regulate other functions. These include species like *V. harveyi* (LuxM-LuxN regulating lux genes), *Agrobacterium tumefaciens* (TraR-TraI regulating plasmid conjugal transfer), *Pseudomonas aeruginosa* (LasR-LasI regulating virulence factors), *Erwinia carotovora* (ExpR-ExpI regulating antibiotic synthesis) and *E.*

coli (SdiA - system regulating cell division and expression of virulence factors). In *E. coli*, orphan LuxR-type reseptor SdiA, without the production of the signal protein, acts as a negative regulator of the expression of virulence factors (312-317)**.** In *P. luminescens*, the receptor PluR homologous to LuxR recognizes nanomolar concentrations of endogenous α-pyrones, inducing the transcription of the PCF-operon finally leading to bacterial cell clumping (318, 319).

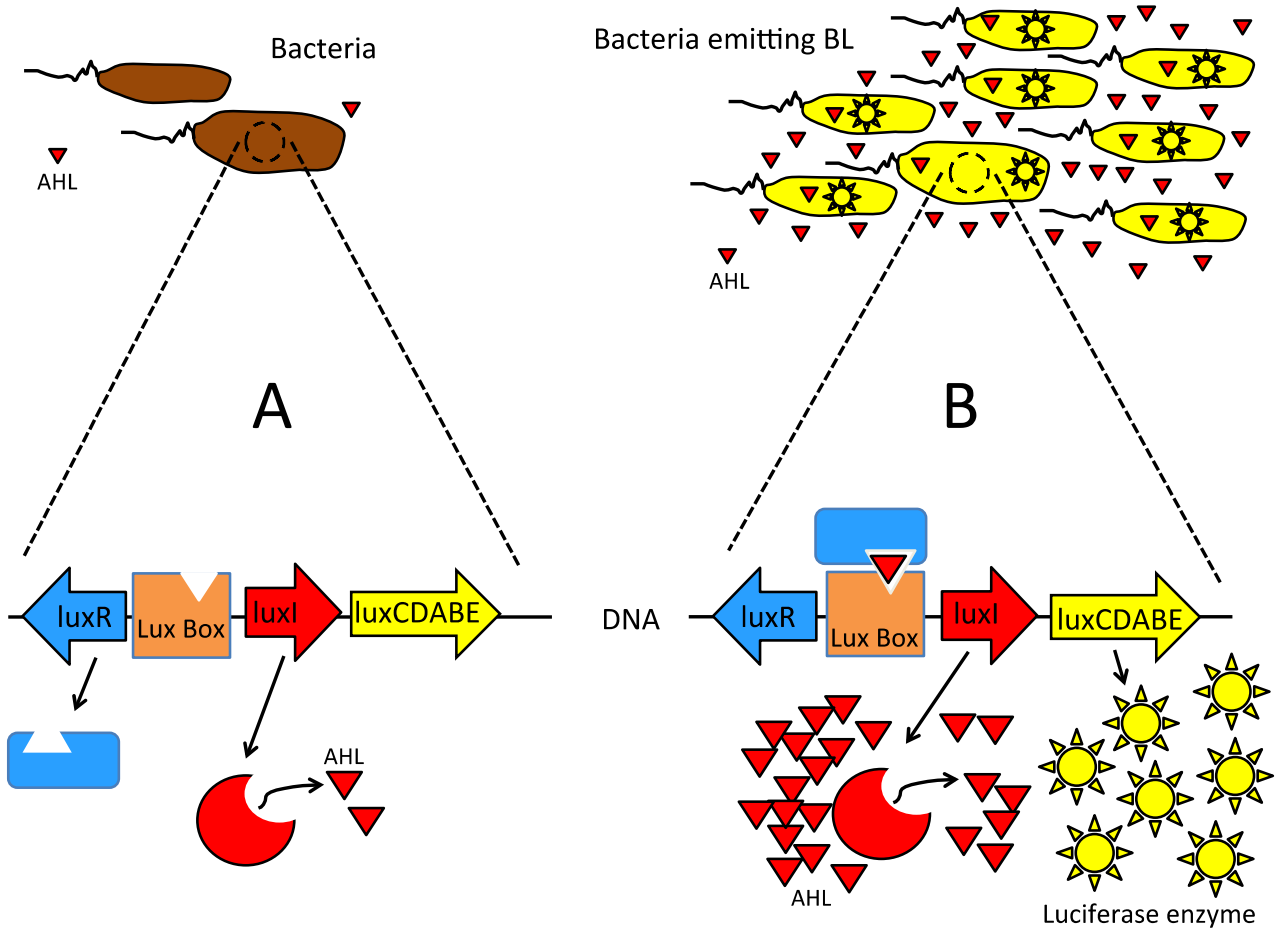


Figure 12: The quorum sensing model of *V. fischeri*: On the left (A) the concentration of acyl homoserine lactone (AHL) is low due to the low number of bacterial cells. On the right (B) the density of bacterial cells has increased and AHL is produced sufficiently (by luxI). AHL is required for LuxR to bind DNA via the 'lux Box'. This activates the luciferase gene luxCDABE producing the bacterial luciferase enzyme, leading to bioluminescence (BL) emission. Simultaneously more AHL is produced (320, 321).

Escherichia coli K-12 pEGFPluxABCDEamp (*E.coli*-lux)

E. coli are a rod shaped, gram (-) bacteria, of the *Enterobacteriaceae* family of bacteria, commonly found in the lower intestine of vertebrates (322-324). The *E. coli* K-12 strain was isolated from an excrement sample of a diphtheria patient in recovery at Stanford University in 1922 (325, 326).
As mentioned earlier, the functional stability of the bacterial luciferase from *P. luminescens* is optimal for laboratory practice. The downside is that the *luxCDABE* gene is situated in a bacterial chromosome under the control of a LuxR-LuxI-type quorum sensing system (Figure 12). This demands high cell density in cultures and assessments, and it is also known that wild type *P.*

luminescens gradually loses its ability to produce BL in laboratory circumstances when removed from the natural symbiotic existence with nematodes. The modified *lux*-cassette *luxABCDE* (249) has thus been transformed into *E. coli* K-12 in a high copy number plasmid *pEGFP* resulting in plasmid *pEGFPluxABCDEamp*. The lux cassette was excised from plasmid pSB2025 (286) (II). The expression of the whole operon (*luxABCDE)*, under the control of Lac promoter, produces a luciferase holoenzyme complex resulting in BL emission without the addition of substrate (II). An enhanced green fluorescence protein (EGFP) gene is placed upstream under the same promoter as the *lux*-gene, and the EGFP is expressed simultaneously with luciferase. In order to maintain the selection pressure, the *pEGFPluxABCDEamp* plasmid also includes the *amp*-gene, encoding β-lactamase, enabling *E. coli*-lux to degrade specifically ampicillin but also to some extent other β-lactam antibiotics, like penicillin and amoxicillin (327-330).
E. coli-lux provides a convenient means for assaying the antimicrobial activity of different agents like toxins, other chemicals and immune systems like the serum complement system and the neutrophil activity. This method has been used to study fish, (I) human (II) and bat (III) complement activity, bactericidal properties of the haemolymph from species *Bombyx mori* and *Galleria mellonella* (IV) and it has been employed to untangle the mechanisms of neutrophil activities in bacterial killing (V, VI).

2.5.3 Neutrophil chemiluminescence assay

Neutrophils emit small amounts of electromagnetic radiation following the ingestion of microorganisms. The energy can be detected as light by sensitive photomultiplier tubes such as those in liquid scintillation counters and luminometers. During RB, H_2O_2, superoxide radicals and singlet oxygen are generated. Singlet oxygen, a highly unstable ROS, combines with bacteria or other intralysosomal elements to form electronically unstable carboxyl groups. As these groups relax to their ground state, light energy is emitted. This process has been termed chemiluminescence (CL), and it forms the basis of an important assay of neutrophil function. It requires all steps prior to actual bacterial killing to be intact (331-338). The oxidative steps in biochemical pathways present in neutrophils generate the CL, which is easily detected in a luminometer. In the test system, isolated neutrophils or whole blood dilutions are incubated in the presence of an ingested particle, e.g. latex particles, zymosan (the cell wall preparate of *Saccharomyces cerevisiae*) or bacterial cells in the luminometer vial, mainly in microtiter wells (331-338). Luminol or lucigenin, intermediate fluorescent compounds, are added to intensify the light emission. The resultant CL emission is directly linked to the activity of neutrophils (335, 336, 339-341). Luminol derived CL (emission max ~ 450 nm) is linked to the release of myeloperoxidase (MPO) from the azurophilic granules of the phagocytes (342). Lucigenin (CL emission max ~ 480 nm) reacts with the superoxide and indicates the attachment (adhesion) of the target to the neutrophil membrane (343-345).
These methods are very sensitive and, moreover, they can be performed on very small numbers of cells (337, 338, 346). This enables the testing of whole blood dilutions without isolating neutrophils and thus avoiding additional purification steps. It has been shown that in whole blood the major CL emission is due to neutrophil activity, since monocytes, the other considerable CL source, emit only about 50 % of that of one neutrophil, representing nearly 90 % of the whole phagocytic leucocyte population (342, 347).

Cytotoxic activity

The CL measurement of reactive oxidative species in phagocytes is an established method providing a sensitive and fast tool for evaluating the relation of the immune system to stress, disease or the overall pathophysiological state of the organism in the microliter range of blood. This applies especially to neutrophils (339-341) which are exelent indicators in this sence. They rapidly react to the anomalities. It has been reported that for example in suvere burn injuries there is impairments in respiratory burst activity and during the sepsis the complement and adhesion molecule expression is impeded (348, 349). Many cytotoxic agents, such as the myxotoxins described earlier, have an effect on neutrophil activity and enable the use of these cells in toxicological assessments (335-341, 346). This also applies to the toxicological assessments where the measured toxic agent is added straight to the CL reaction effecting the light emission. Cytochalasin D (see mycotoxins section) is a textbook example of how mycotoxins affect neutrophils, decreasing the CL signal in a dose-dependent manner. The Fusarium toxin MON (see mycotoxins section) reduced the neutrophil activity of the test rats in a dose and temporal dependent manner (VII).

3. THE AIMS OF THE STUDY

1. The aim was to study the kinetical and real time based characteristics of the recombinant *E. coli*-lux construct, study the viability of this bacterial strain and gain knowledge from the antimicrobial properties of different chemical agents like solvents, toxins and antibiotics (II).

2. The second aim was to determine the kinetical parameters of bacterial killing by the serum complement system utilizing the real-time based bioluminescence approach and *E. coli*-lux as a probe (I, II, III, IV).

3. The third aim was to determine the killing parameters of neutrophils and especially the myeloperoxidase-H_2O_2-Halide system in cell-free systems *in vitro* and *in vivo* by characterizing pathogen killing by the intact neutrophils. The aim was to exploit a variety of different MPO-system inhibitors and antagonists as well as to include luminol and luciferease amplified chemiluminescence (CL) measurements (V, VI, VII).

4. The fourth aim was to exploit the developed applications, capable of practical toxicological assessments, by using both *E. coli*-lux and human neutrophils as probe cells (VII, VIII).

4. MATERIALS AND METHODS

Original publications I-VIII contain more individual and detailed information on the materials and methods used in the studies presented in the results and discussion section.

Bacterial strains and plasmids (II)

The plasmid *pEGFPluxABCDEAmp*, assembled by ligating the modified bacterial luciferase genes *luxABCDE* (from plasmid *pSB2025*, by means of NcoI and PstI restriction enzymes) into *pEGFP* (Clontech, Saint-Germain-en-Laye, France), was transformed to *Escherichia coli* K-12 strain M72 (Sm^R*lacZ*(Am)ΔbiouvrBΔtrpE42[λn7(Am)N53(Am)ca857ΔH1]) (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) by electroporation with the Bio-Rad Gene Pulser system (Bio-Rad, Richmond, CA, USA). The new construct, designated as *E. coli*-lux, was identified by bioluminescence (BL), fluorescence emission and ampicillin resistance (II).

The *Photorhabdus luminescens* subsp. *luminescens* CCM 7077^T, originally from the Czech Collection of Microorganism (Brno, Czech Republic), was kindly donated by Dr. Pavel Hyršl (Masaryk University, Brno, Czech Republic).

Bacterial culture (I, II, III, IV, V, VI, VIII)

The freezer stock preparation of the bacteria was made by cultivating the *E. coli*-lux in Luria Bertani Broth (LB) (10 g tryptone, 5 g of yeast extract, 5 g NaCl, ampicillin 100 μg/ml pH 7.4) (LB_{amp}) and by incubating in a shaker (250 rpm) at 37 C, until the bacteria was in the stationary phase defined by the turbidity (optical density, OD) measurement at 620 nm (UV-1601 Shimadzu spectrophotometer, Shimadzu corp., Tokyo, Japan). Cells were washed and harvested by centrifugation (3000 rpm, 10 min), resuspended in (LB_{amp}) containing 25 % glycerol and placed in the deep freeze (-80 C) in appropriate containers or directly in the microtiter wells. All *E. coli*-lux cultivation media contained ampicillin (100 μg/ml) to maintain the selection pressure.
For the experimental use, the bacterial cells were cultivated in LB_{amp} and incubated in a shaker (250 rpm) at 37 C until the mid-logarithmic state. Cells were then washed and harvested by centrifugation (3000 rpm, 10 min), resuspended in a particular fresh analysis buffer in order to fine-tune the OD and finally diluted to the desired cell concentration with the particular buffer.
The bacterial concentration of certain OD was determined with colony forming unit assays from serial diluted bacterial suspensions with overnight petri-dish cultivations in LB-agar.

Viability and killing assessments (I, II, III, IV, V, VI, VIII)

The desired bacterial concentration was pipetted into a cuvette (1 ml, 2ml or 4 ml) or into a white microtiter (clear or white bottom) plate well (~50 μl - 300 μl). After this BL and/or OD was measured with a cuvette-exploited device (Hidex triathler, Hidex, Turku, Finland) or with a plate reader [Hidex Sense or Hidex Chameleon] for the required time at 37 C.
Antimicrobial agents were added in desired concentrations. Toxins, serum and neutrophils were tested using Hanks Balanced Salt solution (HBSS) and MPO-H_2O_2-halide system in (77 mM) a phosphate buffer in variable pH.

The dust samples collected with microfiber swipes were diluted in H_2O or dimethyl sulfoxide in 10 mg dust/ml in a sample tube containing the swipe and the specimen dust. For toxicity measurements these were further diluted in HBSS in a desired concentration and mixed with the bacterial suspension in a microtiter well.
Specimens for CFU viability count were collected from a cuvette or a duplicate plate incubated at 37 C, and the specimens were diluted and spread in the LB-agar. Plates were incubated overnight at 37 C after which the colonies were counted. In neutrophil assessments the first dilution for CFU counts were made in distilled water in order to lyse the neutrophils and to release the possible viable bacterial cells from inside the phagocytic cell.

Serum handling and leukocyte separation (I, II, III, IV, V, VI, VII)

For neutrophil activity measurements, peripheral blood samples were collected from healthy adult volunteer donors in ethylenediaminetetraacetic acid (EDTA) tubes, and leukocytes were separated by mixing 1–5 ml of blood to 10–50 ml of NH_2Cl (0.83 %) including 370 mg/ml of EDTA to lyse the erythrocytes. After 15 min of incubation, leucocytes were separated by centrifugation in 400 x g for 10 min. Erythrocyte debris was removed by suction and leukocytes were resuspended in desired cell concentration in HBSS. Further purification was refrained in order to avoid unnecessary stress to the cells.
In the whole blood experiments the samples (EDTA) from humans or rats were diluted to a desired concentration in HBSS without any separation procedures.
In all procedures, cells were counted with a microscope using a Bürker chamber.
For serum experiments, blood was collected from all studied species in serum tubes or capillary pipes and separated by centrifugation. Serum dilutions were made in HBSS and with 10 % ethylene glycol tetra acetic acid (EGTA) when the alternative pathway was in question. Surplus serum samples were stored in the deep freeze at -80 C for further analysis. Haemolymp was collected correspondingly from killed insects.

Chemiluminescence assessment (V, VI, VII)

Amplified CL assay was operated by pipetting luminol (Sigma, St. Louis, MO, USA) or lucigenin (Sigma, St. Louis, MO, USA) suspension in the measurement well or a cuvette containing isolated neutrophils or the whole blood dilution and the activator like zymosan (Sigma, St. Louis, MO, USA) or bacterial cells. Luminol and lucigenin can induce CL emission with merely the MPO-H_2O_2-halide system, and CL emission was also studied without neutrophils. The desired inhibitor or toxin was added to the reaction mixture, and after the incubation period, the CL was recorded with a luminometer.

5. RESULTS AND DISCUSSION

5.1 Does the bioluminescence emission of the *E. coli*-lux correlate with the viability?

5.1.1 Batch culture in rich medium (II)

Figure 13 shows a typical batch culture experiment of *E. coli*-lux in LB_{AMP} medium. The inoculum from freezer preparation was made into a flask with 200 ml LB_{AMP} medium containing initially approximately 40 000 CFU/ml. The bioluminescence (BL) and the optical density (OD) were measured and the colony forming unit (CFU) samples collected in 30 min intervals during the 8.5 hours of incubation at 37 C. The BL and OD were measured in a volume of 1 ml (cuvette). The zero OD value was set by using pure LB_{AMP} medium, and the instrumental background BL emission of 267 CPS was subtracted from the BL values.
After seeding the inoculum in the fluid medium, growth starts without the immediate increase in cell number when bacteria cells are introduced and adapted to the fresh culture medium with proper nutrients (II). This adaptation means probable alterations in cell size, concatenation and/or dismantling of the concatenation of the cells (350).

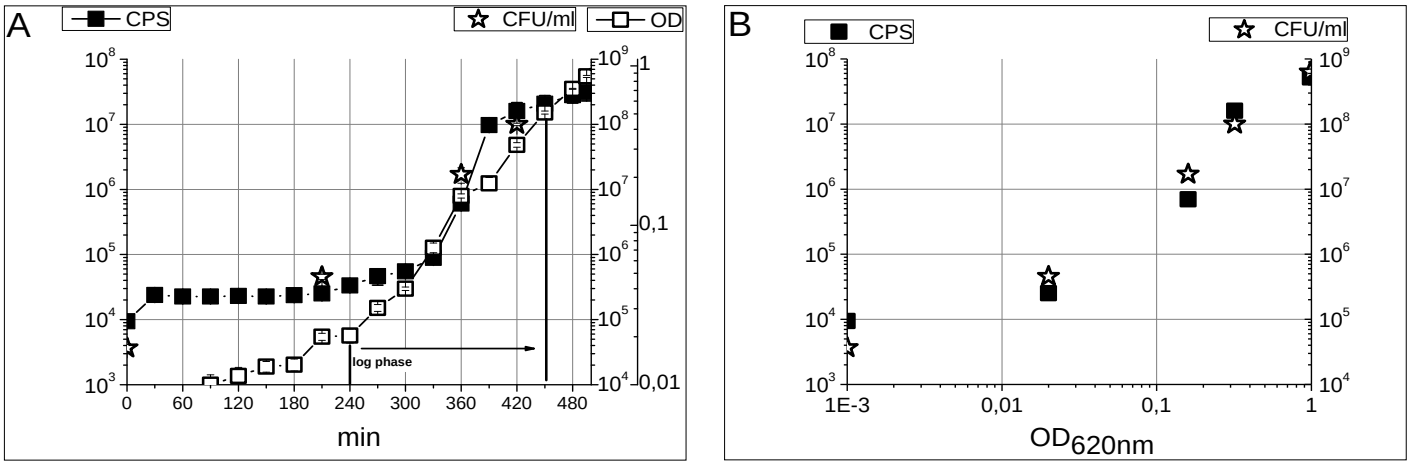


Figure 13: (A) A batch culture experiment of *E. coli*-lux in LB_{AMP} medium performed in a 200 ml flask. The bioluminescence [CPS (counts per second)] (■) and OD (optical density) (□) was measured in a volume of 1 ml in 30 min intervals and five CFU (colony forming unit) (cells/ml) (☆) readings were made during the 8.5 hours of incubation at 37 C. The initial *E. coli*-lux concentration was approximately 40 000 CFU/ml. A log phase is diverged with vertical black lines.
(B) CPS (■) and CFU (☆) are shown as a function of OD during the incubation in the experiment represented in Figure 13A. The average BL emitted by one *E. coli*-lux cell was 0.1 CPS/cell.

BL emission kinetics was committed with increase in the CFU values, and during the whole incubation period, both readings increased approximately 10^4 fold (Figure 13). Figure 13B shows CPS and CFU as a function of OD during the incubation in LB_{AMP} medium at 37 C. The average BL emitted by one *E. coli*-lux cell was approximately 0.1 CPS/cell. The linear part of the figure begins after 0.01 OD (Figure 13B), and this OD value seems to be the detection limit (approximately 10^5 *E. coli*-lux cells/ml) of the used cuvette photometer, with the light path of 10 mm. Values below 0.01 OD are artifacts due to noise signal and do not represent real turbidity data. This inability to detect low bacterial concentrations is the reason why OD signal elevation during the whole incubation

period was only 10^2 fold, though the turbidity should also correlate directly with the increase of the CFU values (72-74).
The logarithmic growth phase [1-3] starts after 240 min of incubation and is preceded by an acceleration phase [1-3] starting at 120 min. The lag phase is not visible in the turbidity data, as before 120 min the OD signal is lower than 0.01 OD. During the log phase, which is decelerated after 450 min, the average generation time of *E. coli*-lux strain during the log phase was 40 minutes.
The BL signal demonstrates a significant increase (from 2 x 10^5 to 10^7 CPS) after 330 min of incubation (Figure 13 A). The external addition of a substrate aldehyde, like decanal, during the steady signal emission of the first hours of incubation caused a transient sharp increase in the BL signal (data not shown), suggesting that the availability of fatty aldehyde was a limiting factor for BL production (249, 260, 273-275).
Mid-logarithmic *E. coli*-lux cells were further used in killing experiments, since during the exponential growth phase the microbe population is at its most uniform and viable in terms of chemical and physiological properties (72, 73, 351) (I – VII).

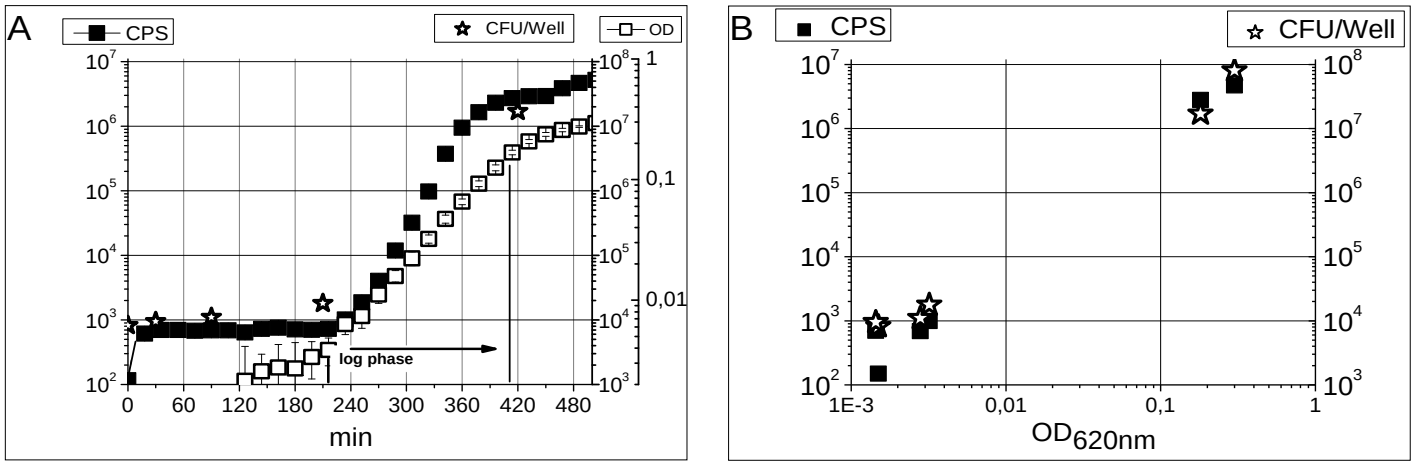


Figure 14: (A) A batch culture experiment of *E. coli*-lux in LB_{AMP} medium performed in the plate reader in 200 µl in the well (transparent bottom) of the white microtiter plate. The bioluminescence [CPS (counts per second)] (■) and OD (optical density) (□) were measured at regular intervals, and six CFU (colony forming unit) (cells/well) (☆) readings were made during the 10 h incubation at 37 C. The background signals of LB_{AMP} medium 69 CPS and 0.045 OD were subtracted from the measurement values. The initial *E. coli*-lux concentration was 8000 CFU/Well (40 000 CFU/ml). The CPS and OD values are shown as the mean ±SD of measurements of three parallel wells.
(B) CPS (■) and CFU (☆) are shown as a function of OD during the incubation of the culture represented in Figure 14A. The average BL emitted by one *E. coli*-lux cell was approximately 0.07 CPS/cell.

When similar measurements were made in the volume of 200 µl in LB_{AMP} in a microtiter well measured with the newest model, Hidex Sense, plate reader (Figure 14), the BL emission follows the same pattern as in the higher volume (Figure 13). The inoculation was made so that the concentration of the viable bacteria was 40 000 CFU/ml (8000 cells/well), similar to the measurement presented in Figure 13. In the plate reader there was a background signal of 69±5 CPS (n=20) and 0.045±0.007 OD (n=20), and these average values were subtracted from CPS and OD readings respectively.
During the whole incubation period, the BL signal and CFU viability both increased approximately 10^4 fold. In the microtiter well in a volume of 200 µl (Figure 14B) the CPS/cell ratio was 0.07 CPS/cell. BL emission seemed to be committed to the CFU in both experiments. The increase in the OD readings was 10^2 fold. The detection limit of the photometer of the Hidex Sense plate reader was approximately 2 x 10^4 CFU/well corresponding to 0.003 OD. OD values beneath this were artifacts

also indicated by the high noise to signal ratio before the 210 min time point represented in Figure 14A.
However, above the detection limit the turbidity data manifested advisable dynamics in both experiments (Figure 13 and 14), and above these limits the absorbance measurement is applicable to the viability assessments. In Figure 18A, the log phase starts after 210 min of incubation and ends after 3.5 h (Figure 14A). During this time, the BL signal increases significantly. The same phenomenon was observed in the case of the 200 ml culture (Figure 13A).

It is noteworthy that in the microtiter well in the volume of 200 µl the theoretical light path was 3/5 of that in the cuvette, and according to Lambert Beer's law this should also correlate with the ratio of $OD_{plate\ reader}/OD_{cuvette}$. However, inside the detection limit this ratio was 2/5 on the average (352).
Actually, due to meniscus variations caused by the buffer composition, the actual light path in the well is shorter and there is no reliable way to calculate the liquid path length solely by the added volume. The path length has to be measured separately from each well and this value used to normalize the measured absorbance to 10 mm path length (352).

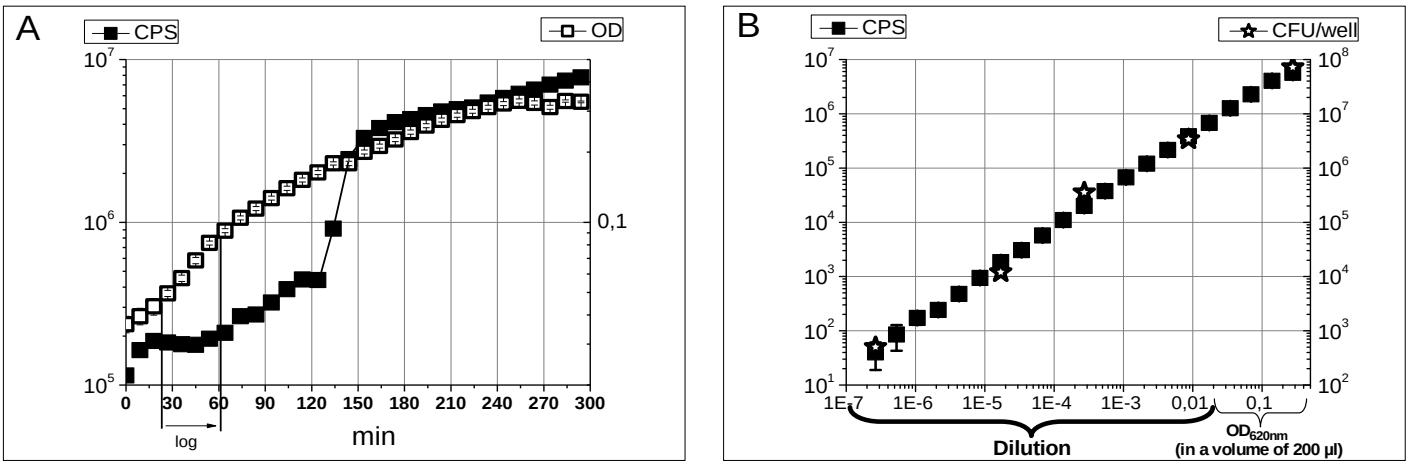


Figure 15: (A) The *E. coli*-lux culture made in the plate reader in LB_{AMP} 200 µl from the log phase cells. OD and BL were recorded during 5 h of incubation at 37 C. The bioluminescence [CPS (counts per second)] (■) and OD (optical density) (□) were measured at regular intervals during the 300 min of incubation at 37 C. **(B)** CPS (■) and CFU (colony forming unit) (cells/ml) (☆) are shown as a function of OD during the incubation in the experiment represented in Figure 15A. There was a background signal of 128 ±8 CPS (n=20) and 0.03±0.007 OD (n=20), and these average values were subtracted from CPS and OD readings respectively. The average BL emitted by one *E. coli*-lux cell was approximately 0.09 CPS/cell. CPS and OD values are shown as the mean ±SD of the measurements of three parallel wells.

Other experiments presented further in this book are measured with the older Hidex Chameleon plate reader. There was a background signal of 128±8 CPS (n=20), and this average value was subtracted from CPS readings. The detection of BL was similar and the BL kinetics comparable with the newer measurement techniques. The detection limit and the dynamic range of the turbidity measurements, however, were much weaker. The background signal was 0.03±0.007 OD (n=20), and the actual detection limit 0.02 OD corresponding to 4 x 10^6 cells/well. The Hidex Chameleon plate reader is not ideal for batch culture experiments (II). However, above the detection limit the measurement dynamics was also passable.

The dilution series was prepared from late log-phase cells, diluted until the OD in the plate reader was near the detection limit. The volume of 200 µl of this *E. coli*-lux suspension in LB_{AMP} was pipetted into the microtiter well and OD and BL was recorded during 5 h of incubation at 37 C (Figure 15A). There was no significant lag phase and cells went into the log phase after 15 min of inoculation lasting approximately 40 min with the gain of one generation. The BL elevation (Figure 15A) begins during the log phase, the phenomenon already demonstrated in the batch culture experiment introduced in Figures 13A and 14A.
Figure 15B represents BL and CFU (not shown in Figure 15A) as a function of OD during the incubation of the culture introduced in Figure 15A. The detection limit of the plate reader seemed to be 0.02 OD, and the lower values are from the dilution series. The average BL emitted by one *E. coli*-lux cell was 0.1 CPS/cell (II).

5.1.2 Batch culture in minimal media (II)

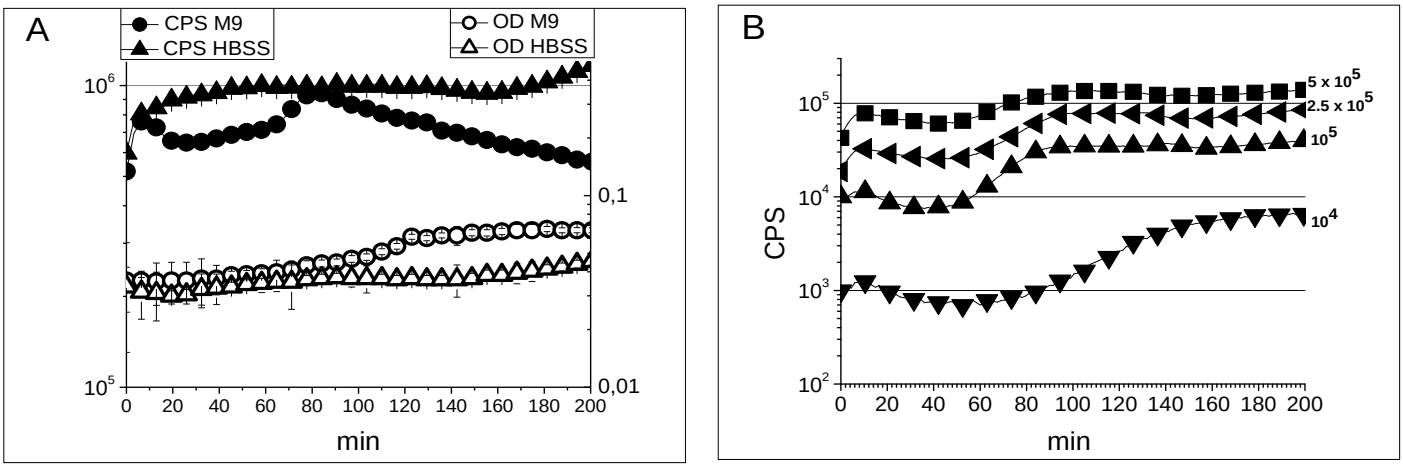


Figure 16: (A) The bioluminescence [CPS (counts per second)] [(●) M9, (▲) HBSS] and OD (optical density) [(o) M9, (Δ) HBSS] of the E. coli-lux were observed during 200 min of incubation in the plate reader in 200 µl at 37 C. The inoculation was approximately 5 x 10^6 E. coli-lux cells/well. There was a background signal of 128 ±8 CPS (n=20) and 0.03±0.007 OD (n=20), and these average values were subtracted from CPS and OD readings respectively. **(B)** The incubation of different dilutions from the HBSS culture shown in Figure 16A [(■) 1/10, (◀) 1/20, (▲) 1/50, (▼) 1/500] 200 µl HBSS buffer and incubated 200 min in the plate reader at 37 C. CPS and OD values are shown as the mean ±SD of measurements of three parallel wells

The growth rate of the bacterial population depends on the composition of the growth media, and having determined the correlation between the BL emission and the number of viable *E. coli*-lux cells during the batch culture in rich medium, the BL production and the growth of the *E. coli*-lux was subsequently observed in various minimal media, such as M9 and hanks balanced salt solution (HBSS), presented in Figure 16.
Because neither of these media supported intensive growth, inoculation was prepared from the dilutions from log phase LB cultivation. After dilution, cells were washed and resuspended in $M9_{AMP}$ and $HBSS_{AMP}$ mediums in 200 µl in microtiter wells, initially containing approximately 5 x 10^6 *E. coli*-lux CFU/well.

The bacterial growth in minimal medium during the 200 min of incubation was marginal (Figure 16A). The BL signal in $M9_{AMP}$ was unstable, but in $HBSS_{AMP}$ it remained steady through the whole incubation period. The CFU values (data not shown) reveal that the number of cells in $HBSS_{AMP}$ also remains steady (5×10^6 *E. coli*-lux CFU/well). The CFU in $M9_{AMP}$ medium after 200 min was 2×10^6 *E. coli*-lux CFU/well, indicating the gradual loss of viability during the incubation period. It was noteworthy that the BL signal also decreased in the same ratio.
Average BL emitted by of one *E. coli*-lux cell, was 0.1 CPS/cell. Figure 16B presents the $HBSS_{AMP}$ incubations made from the dilution series from the $HBSS_{AMP}$ incubations.

In conclusion, the *E. coli*-lux based BL emission system seems to be applicable in kinetical and real-time definition of bacterial viability. Turbidity measurement also provides such an assay but with much weaker measurement dynamics, resulting from the weak sensitivity of the plate reader photometer. In the older plate reader, the detection limit was at the level of 10^6 bacterial cells in the well, requiring 10^4 times higher numbers of bacterial cells compared with the BL measurement (II). However, the newer plate reader obtained higher sensitivity and it can also be utilized in batch culture experiments.
Figures 13, 14, 15 and 16 demonstrate that the BL emission of *E. coli*-lux is directly linked to the number of viable bacterial cells, both in rich and in minimal media with efficient measurement dynamics, capable of detecting from as few as 10^2 bacterial cells/well to as many as 10^8 cells/well, with 0.1 CPS/cell ratio (II).
Since the HBSS sustained stabile cell viability (both BL emission and OD readings properties), it seemed to be the ideal experiment buffer, providing cells with water and inorganic ions, while maintaining a physiological pH and osmotic pressure. It was shown in our experiments and in literature that bacteria in the log phase and simple salt medium (buffer and HBSS) are more susceptible to toxins and other antimicrobial effectors than in rich media (351).

5.2 Antimicrobial study

Killing E. coli-lux by using ethanol and polymyxin B (II, VIII).

It was observed in the case of the batch culture in minimal media M9 (Figure 16A) that the diminishment of the BL signal correlated with the degradation of CFU, i.e. the number of naturally deceased bacterial cells was linked to the decrease of the BL signal. Would the same correlation still be valid if the extrinsic antimicrobial agent was added?

The antimicrobial assessing was started by incubating *E. coli*-lux in the presence of ethanol (Figure 17) and antibiotic polymyxin B (PmB), which is a cationic protein, derived from the bacterium *Bacillus polymyxa* (177) altering the permeability of the bacterial cell membrane and finally disrupting the cell (285).
Two distinct antimicrobial effects, the bacteriocidic and bacteriolytic effects (134) (I, II) introduced in literature review section in Figure 6, were observed in ethanol incubation (Figure 17) and one in PmB (Figure 18). First is the bacteriocidic effect, denoting the loss of viability of the bacterial cells, which can be observed using all three techniques (BL, OD and CFU) presented in Figure 17, when *E. coli*-lux was incubated in the presence of 20 % ethanol or 1.25 μg/ml PmB. The reduction of the turbidity indicates the bacteriolytic effect (Figure 17B), leading to lysis of the target cells. The bacteriolytic effect is always a bacteriocidic effect, but not vice versa, since lysis is not mandatory for the loss of viability, i.e. the loss of viability may result in the reduction of BL signal and CFU count but not necessarily the reduction of OD readings if the cells remain intact (134) (I, II). The

third antimicrobial effect is bacteriostatic (134) (I, II), and since the HBSS did not support any growth during the incubation period, this effect is not visible.
The fourth effect is not an antimicrobial reaction per se but stimulation (72) of metabolism and growth caused by the antimicrobial agents exposed in small enough concentrations. This effect is represented in Figure 17, where the lowest concentration of ethanol 1.25 % caused BL and CFU signal elevation above the reference.

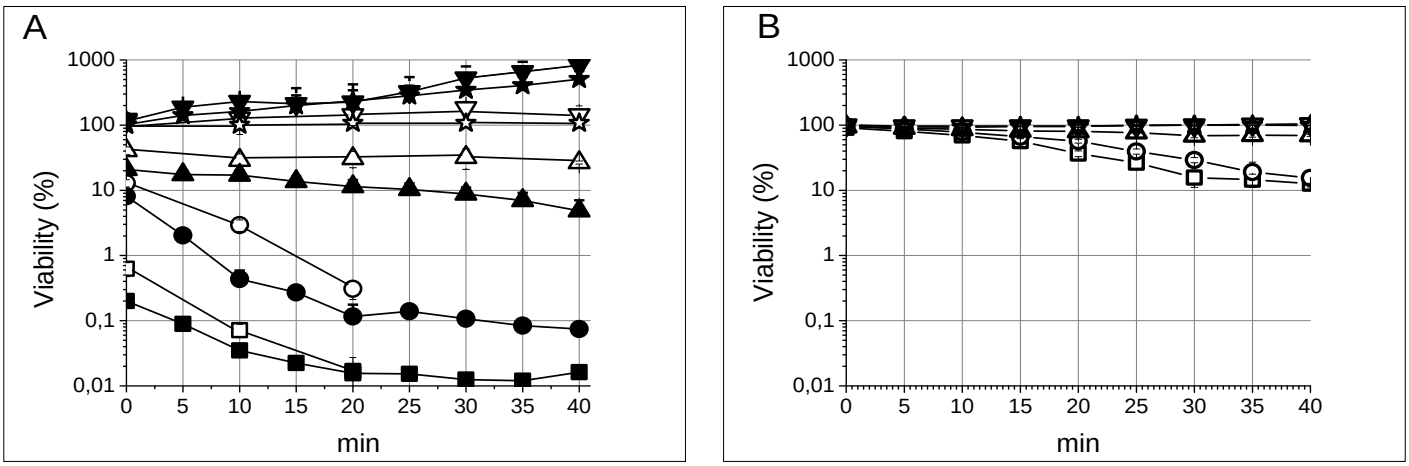


Figure 17: The viability of 6 x 10^6 *E. coli*-lux incubated in 200 µl HBSS at 37 C in the presence of 20 % (square), 10 % (circle), 5 % (triangle up), 1.25 % (triangle down) and 0 % (star). **(A)** The viability calculated from bioluminescence (black symbols) and colony forming unit readings (open symbols). **(B)** The viability calculated from optical density (OD) readings (open symbols). The 100 % is set as the initial reference value with no ethanol (II). CPS and OD values are shown as the mean ±SD of measurements of three parallel wells.

In the presence of ethanol (Figure 17) and PmB (Figure 18), the antimicrobial effect is visible in all three methods used at high enough concentration. The decrease in BL signal correlated with CFU (Figure 17A). The decrease in OD (Figure 17B) was apparently smaller due to a more restricted dynamic range.
It was also noteworthy that, during the bacteriolytic reactions, the plate reader OD readings never decreased under the detection limit (regardless of the CFU decreasing below 4 x 10^6 cells/well). This was probably caused by cell debris cumulating in the measurement well.
The results can be plotted as a dose-dependency curve presented in Figure 18B, where the maximum killing effect after 30 min of incubation is plotted as procentual killing against the concentration of the antimicrobial agent. The 100 % killing was defined as the BL level reaching the background signal of 128 CPS on the plate reader. The 0 % of killing is the level of the reference signal from the bacterial well without exposure to antimicrobials. From this we can define the EC_{50} (effective concentration) or EC_{90} values, i.e. the concentration of antimicrobial agent killing 50 % or 90 % of the bacterial population at a given time point, respectively. According to Figure 18B, EC_{50} for PmB after 30 min of incubation is 0.04 µg/ml or 0.045 µg/ml, estimated from CFU and BL measurements respectively. The EC_{90} values were 0.06 µg/ml and 0.09 µg/ml respectively.
When using rapidly affecting antimicrobial agents like ethanol and PmB, the decrease in the BL signal follows the ratio of the decrease of CFU readings (II). The luminometric approach seems to provide a reliable estimation of *E. coli*-lux viability and killing also in the presence of the extrinsic antimicrobial agent.

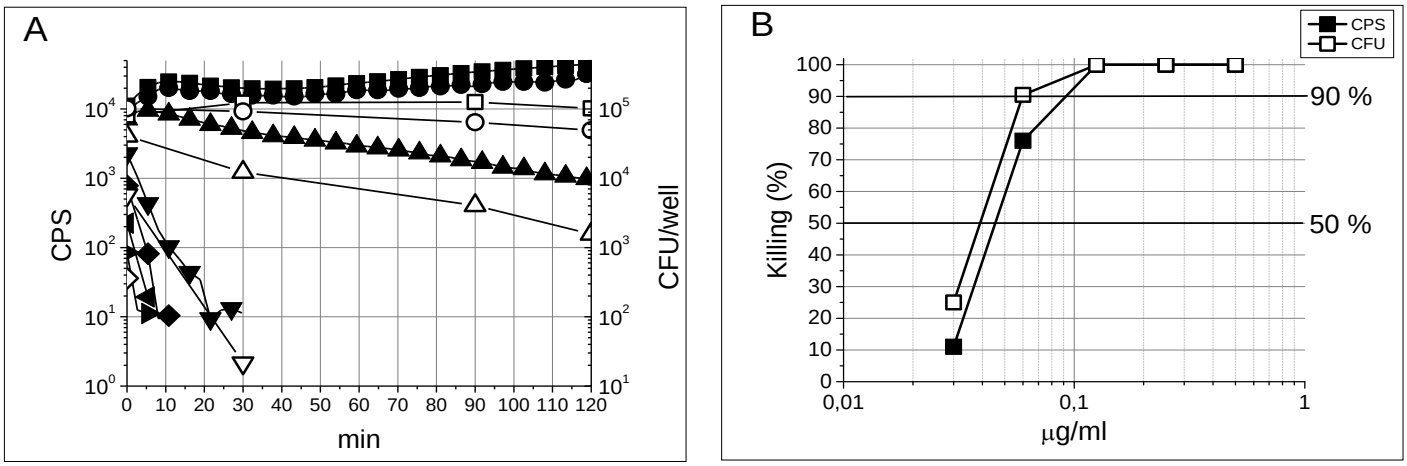


Figure 18: (A) The effect of the polymyxin B against 10^5 *E. coli*-lux cells and the bioluminescence [CPS (counts per second)] from wells containing 0 μg/ml (■), 0.03 μg/ml (●), 0.06 μg/ml (▲), 0.125 μg/ml (▼), 0.25 μg/ml (◆), 0.5 μg/ml (◀) and 1,0 μg/ml (▶) and CFU (colony forming unit) (cells/100 μl) 0 μg/ml (□), 0.03 μg/ml (○), 0.06 μg/ml (△), 0.125 μg/ml (▽), 0.25 μg/ml (◇), 0.5 μg/ml (◁) and 1.0 μg/ml (▷) of polymyxin B in 100 μl HBSS at 37 C. **(B)** The dose-dependency of the polymyxin B after 30 min of incubation to the killing measured from CPS (■) and CFU (□) values. The 100 % killing was defined as the CPS or CFU readings declined to the level of the background signal. CPS values are shown as the mean ±SD of the measurements of three parallel wells.

5.3 Innate immune systems

The serum complement system and neutrophil activity (phagocytosis) are the main tools utilized by the immune system to restrict and kill pathogens invaded to the vertebrate system. The actual quantification of antimicrobial properties, the real-time kinetics of the bacterial killing, and the killing capacity among many other qualities of these vital systems was studied (I, II, III, IV, V, VI, VIII).

Previously, these parameters of the innate immune system were mainly studied with plate counting assays. Now *E. coli*-lux provided an alternative method with many advantages compared to conventional ones, like the availability of real time data, the use of small bacterial numbers in the reaction and short incubation periods.

5.3.1 The complement system (I, II, III, IV)

The cell death kinetics of the complement system was assessed (Figure 19) by adding 10 % human serum to the measurement well containing 6 x 10^6 *E. coli*-lux cells in 200 μl HBSS buffer at 37 C. EGTA chelates free Ca^{2+} ions in the solution inhibiting the classical (Figure 19A) and lectin pathways (LP), leaving only the alternative pathway (AP) intact (Figure 19B) (I, II).

After mixing the bacteria with the serum, there was a period (**a**) before the signals (CPS and OD) started to decrease. This was designated as an activation phase, during which the pathways were activated and the membrane attack complex (MAC) was formed before the onset of killing (353).

In Figure 19A, (**a**) was 17 min and with EGTA serum 86 min (Figure 30B). The actual killing represented in Figure 30A was due to the activation of the classical pathway (CP) (353), since the LP is reported to be in a minor role or even inactive in many cases (353). As reported, CP is a fast operator and the time period from the activation to mac formation and the onset of killing was four times shorter than that of AP.

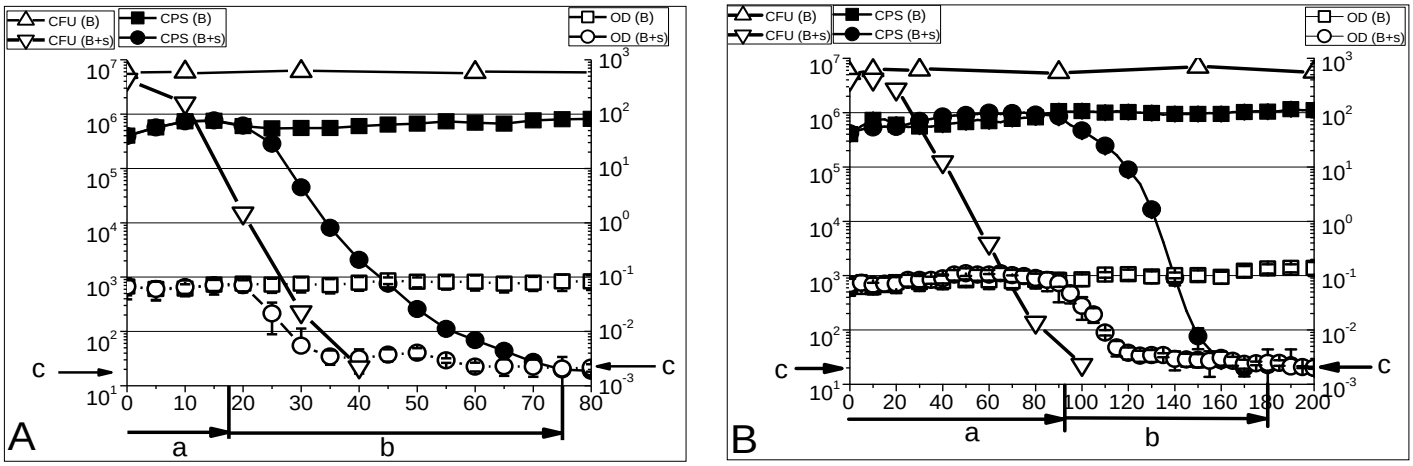


Figure 19: The viability of 6 x 10^6 *E. coli*-lux incubated in 200 µl HBSS at 37 C in the presence of **(A)** 10 % intact human serum and **(B)** 10 % of EGTA treated human serum. The bioluminescence [CPS (counts per second)] *E. coli*-lux (■) and *E. coli*-lux + serum (●), OD (optical density) *E. coli*-lux (□) and *E. coli*-lux + serum (○) and CFU (colony forming unit) (cells/well) *E. coli*-lux (Δ) and *E. coli*-lux + serum (∇) readings were measured at regular intervals during the 200 min. Arrows: **a**: Activation phase (min), **b**: Lytic phase (min) and **c**: Residual level (CPS, OD, CFU)

After the onset of killing, in both pathways, the CPS and OD signals started to decline simultaneously. This indicates bacteriolytic activity, and the phase (**b**) was designated as a lytic phase. In CP, it lasted 58 min (Figure 19A) and in AP 84 min. In CP, the killing by the entire pathway (**a** + **b**) was 75 min and in AP 180 min. The residual level (**c**) presented viable cells in the measurement well. From the residual and primary number of cells, the capacity of killing for the particular serum dilution can be obtained (I, II). Figure 19 shows that the killing capacity was the same in both pathways.

It is notable that no activation phase was observed in CFU kinetics (Figure 19). In the CFU assay the plates are incubated overnight and presumably the activation phase continued overnight. This was visible in Figure 16 at zero incubation (0 min), where the diminishment of the CFU viability in plates was already 25 % (II).

Since the length of the activation phase differentiates CP from AP, we added anti-C1s and anti-C2 antiserum in the reaction. Both antisera prolong the activation phase from that of the CP (22 min) to that of the AP (42 min) (I)**.** These results also suggest that the LP was not active or had a minor role, since C2 is an important component of this pathway. By using antisera, monoclonal antibodies or component depleted sera, we are able to reveal anomalies, interindividual differences and complement component deficiencies with the *E. coli*-lux method (I, II).

The conventional method for the analysis of complement system activation is the total haemolytic assay (CH_{50} and AH_{50} values for CP and AP respectively) (284, 354).

These values indicate the capability of the complement system to lyse 50 % of sheep erythrocytes during 90 min. In a real-time based application, like the BL approach presented in Figure 16, the differences between parameters **a**, **b** and **c** and EC_{50} or EC_{90} can easily be determined and compared. Moreover, it enables the assessment of multiple serum samples and dilutions simultaneously on the

same microtiter plate (96 wells) and, moreover, reaction kinetics is readable and comparable throughout the whole incubation period (I, II).
The characterization of antimicrobial parameters of serum was extended to some other species, among them rainbow trout, pig and bat (I, III). We characterized the suppressive effects of the organic tin compounds to the complement system of the bat by testing the serum collected from populations of the bat *Myotis daubentonii* naturally exposed to tin compounds (III).
Arthropods do not utilize a similar complement system to vertebrates but have a group of antimicrobial peptides in their haemolymph (the liquid in their open circulation system), acting in a similar host defense role to the complement system in vertebrates. The *E. coli*-lux, alongside *P. luminescence*, the source of the lux-gene, was used to test the antimicrobial effectiveness of the haemolymh from the larvae of the species *Bombyx mori* and *Galleria mellonella*. Similar antimicrobial functions to those existing in the vertebrate serum were observed. Though these two phylogenetic diverse systems do not bear any structural similarities, they seem to constitute a common functional model and location (IV).

5.3.2 Neutrophil activity (V, VI, VII)

Myeloperoxidase (MPO) system

The conventional view in the literature is that the MPO-H_2O_2-halide system would be the main killing mechanism during the respiratory burst (RB) by neutrophil phagocytosis. By using the BL approach, the complicated mechanisms of the numerous variable factors of RB were assessed (V, VI) (14-17, 25, 54).

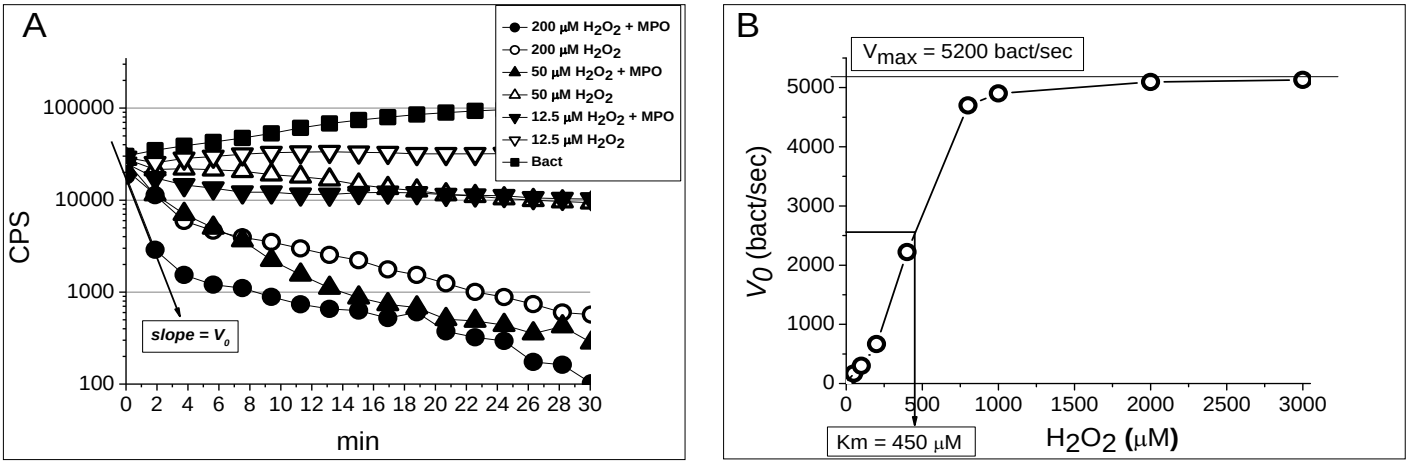


Figure 20: (A) Bioluminescence (CPS) kinetics of *E. coli*-lux (2 x 10^5 cells) (black square) incubated in the presence of different concentrations of H_2O_2 [12.5 μM (triangle down), 50 μM (triangle up), 200 μM (circle)] with (open symbols) and without (black symbols) 7 nM MPO in a plate reader in 100 μl phosphate buffer with 154 mM chloride (pH 7) at 37 C. Slope $\boldsymbol{V_0}$ represents the rate of the reaction. **(B)** The Michaelis-Menten kinetics defined from the killing kinetics of the *E. coli*-lux by using the V_0 values (arrow in Figure 20A).

The pH varies between 5 to 8 during RB and phagocytosis; tough the actual bacterial killing takes place at a higher pH (pH 7.8) (25, 61-64). There are several available halide ions, Cl^-, Br^-, I^- and F^-,

and SCN^- pseudo halide ion, included and, moreover, there are numerous both oxidative and non-oxidative bactericidal agents present in a phagolysosome. The results and experiments described here were made with Cl^-, which was reported to be the most abundant halide in phagolysosome.

Figure 20A shows the H_2O_2 killing kinetics and the enhancement of the antimicrobial reaction when MPO was added to the reactions at pH 7. This kinetics (Figure 20A) seemed to obey the characteristics of Michaelis-Menten kinetics (355, 356) (Figure 20B), and $\boldsymbol{V_{max}}$ and $\boldsymbol{K_m}$ could be determined provided that v_0 was defined as a number of killed bacterial cells in a time unit. If $\boldsymbol{v_0}$ is plotted against substrate concentration **s** (H_2O_2 concentration), the curve represents the section of rectangular hyberbola and by substituting $\mathbf{v}=\boldsymbol{V_{max}}/\mathbf{2}$ becomes $\mathbf{s}=\boldsymbol{K_m}$ (450 mM H_2O_2). These values can be defined to the different amount of MPO and bacteria used in reaction.

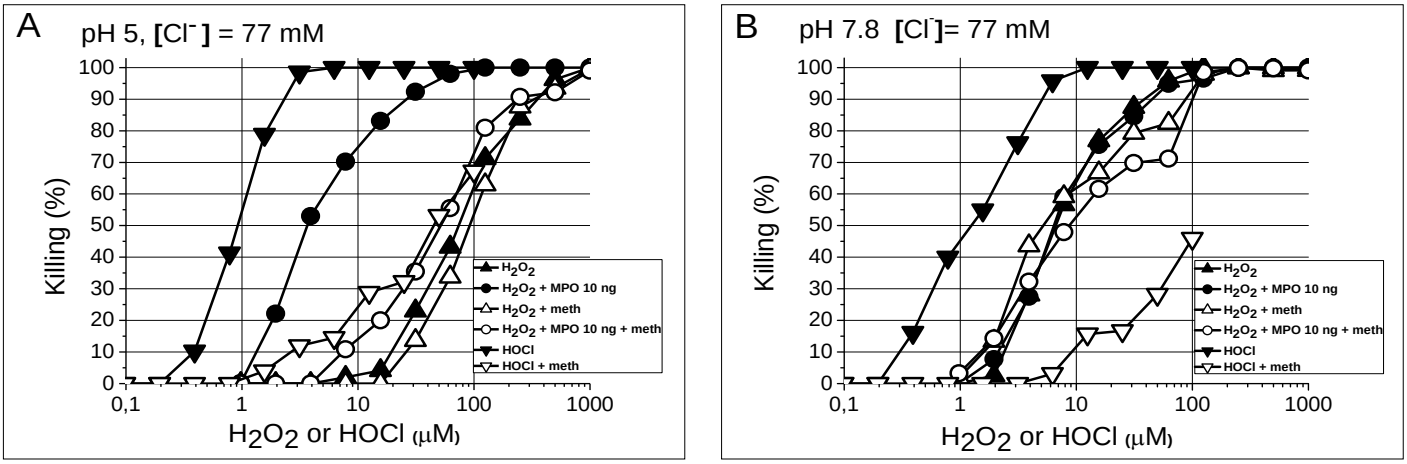


Figure 21: The dose-dependency after 30 min of incubation of H_2O_2 (triangle up), H_2O_2 + 0.7 nM myeloperoxidase (MPO) (circle) and HOCl (NaOCl) (triangle down) in (**A**) pH 5 and (**B**) pH 7.8 phosphate buffer with 77 mM NaCl against 10^5 *E. coli*-lux cells in 100 µl phosphate buffer at 37 C. Open symbols show the same results in the presence of 1 mM methionine. The 100 % killing was defined as the CPS reading declined to the level of the background signal and the 0 % of killing as the CPS reading from the bacteria with no toxin added.

The dose response (Figure 21) was gained from the reaction where *E. coli*-lux (10^5 cells) was incubated in the presence of different concentrations of H_2O_2 with and without 0.7 nM MPO at different pH (pH 5 and pH 7.8). HOCl was also tested in different concentrations (V, VI).
At pH 5 the addition of MPO enhanced the killing significantly (Figure 21A), but when pH was increased to pH 7.8, being the real pH value during the actual killing process inside the phagolysosome (25), the added MPO did not enhance the H_2O_2 killing, i.e. there was no chlorination capacity available (Figure 21B). Inactivation means that HOCl is not produced, because Figure 21 shows that HOCl is pH was a prominent microbicide at both pH values presented.
Bacteria presumably have "safe-groups", like methionine (Figure 21), cysteine and glutathione, containing a sulfhydryl group (R–SH) possibly scavenging the radicals produced by the host (357). The effect of the methionine (1 mM) is presented in Figure 31. Methionine scavenged HOCl killing at both pH values. On the other hand, methionine did not inhibit the antimicrobial reaction of H_2O_2 since a ten-fold higher concentration of methionine was needed for the inhibition of H_2O_2 derived killing (data not shown).
During phagocytosis, the serum proteins like albumin also influence the killing process. Inactive serum and serum albumin in different concentrations were tested. Inactivated serum inhibited the MPO-H_2O_2-halide system, H_2O_2 and HOCl killing (data not shown) already in moderate

concentrations, but the bovine serum albumin (20 mg/ml) did not have any effect on the killing (data not shown). The question is in how high concentrations these factors exist in phagolysosome during the actual phase of killing. Active serum factors affect the phagocytic killing of microorganisms by their opsonic and other functions. It is also noteworthy that the active complement system is still operating on the bacterial cell surface during the ingestion and in phagolysosome augmenting the bacterial destruction (2, 3).

Azide, acetaminophen (ACP) and taurine are known inhibitors and modulators of the MPO-H_2O_2-halide system (358, 359) (V, VI). Azide and ACP are MPO inactivators and modulators, respectively, and taurine the HOCl scavenger (V, VI). These compounds were tested with *E. coli*-lux in both the cell free system, which was described earlier, and the cellular system using intact neutrophils (Figure 22).

Azide inhibited the MPO-H_2O_2-halide system at pH 5 but did not have any effect on the H_2O_2 killing. Taurine inhibited the MPO-H_2O_2-halide system at pH 5 and HOCl killing at both pH 5 and pH 7.8. ACP did not inhibit the killing; on the contrary, it enhanced the killing in the cell free system with used concentrations ($\leq$ 1 mM) (data not shown). However, when the same effectors were tested in the cell system, they did not have any effect on the killing of the *E. coli*-lux cells (Figure 22).

Amino acids with R–SH, like methionine, cysteine and glutathione, were also tested in neutrophil reactions. These amino acids did not have any effect on the killing of the *E. coli*-lux cells (data not shown).

The influx of the inhibitors into the neutrophils was confirmed by the CL assay operated simultaneously with the killing assay (Figure 22). Luminol derived CL (emission max ~ 450 nm) is linked to the release of MPO from the azurophilic granules (342). Lucigenin (CL emission max ~ 480 nm) reacts with the superoxide and indicates the attachment (adhesion) of the target to the neutrophil membrane (343-345).

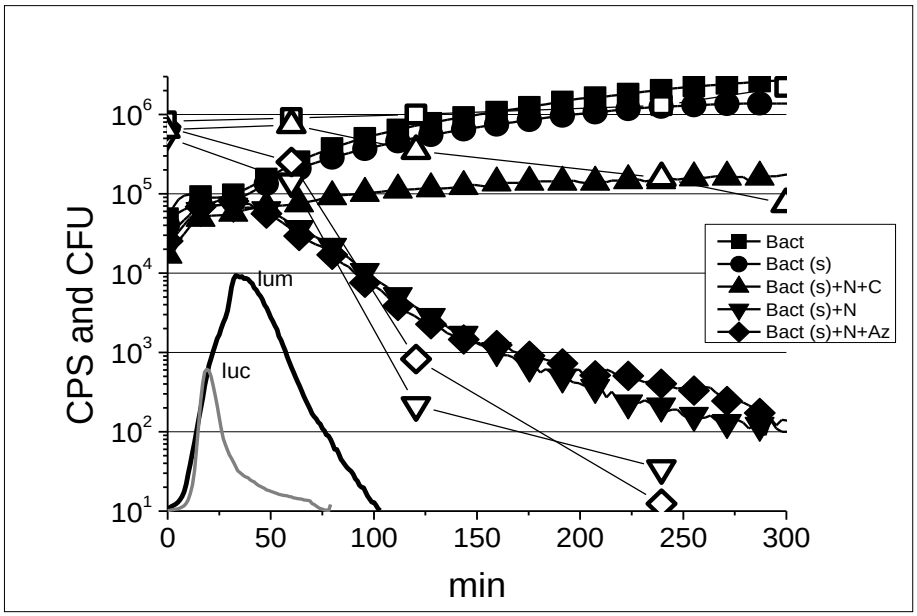


Figure 22: The effects of human neutrophils (10^5 cells) against 10^6 *E. coli*-lux cells. Serum (1 %) was used for opsonization and cytochalasin D (CYT) (10 µM) and methionine (MET) (1 mM) for inhibition. *E. coli*-lux (square), *E. coli*-lux+serum (circle), *E. coli*-lux+serum+neutrophils+CYT (triangle up), *E. coli*-lux+serum+MET (trangle left) and *E. coli*-lux+serum+neutrophils (triangle down). Black symbols represent bioluminescence (CPS) and open symbols colony forming units (CFU). The black line represents the chemiluminescence amplified by luminol (lum) and the grey line by lucigenin (luc).

Figure 22 shows 10^6 *E. coli*-lux cells killing by 100 000 human neutrophils. Cytochalasin D, a mycotoxin produced by *Arpergillus clavatus,* was used in the test system as a phagocytose inhibitor. Cythochalasin D prevents the actin filament function obligatory for ingestion (182, 182, 183).
Figure 22 also shows that azide, MPO inhibitor, did not inhibit the killing by the neutrophils (Figure 22).
Results indicate that the MPO-H_2O_2-halide system is not playing such an established role in the antimicrobial process of neutrophils. The results support the idea presented by Segal et al (25) that MPO-H_2O_2-halide is not the major killing mechanism during phagocytosis, since at pH 7.8 the MPO system is inactivated.
Actually, we have shown that at pH 7.8 the H_2O_2 concentration in phagolysosome, reported in literature to be near 2 μM (46), is enough to kill one bacterial cell without the need for MPO enhancement (**VII**). We presume that the normal bacterial killing utilizes only H_2O_2, and MPO is needed only in an emergency, for example, during severe infections and inflammation when oxygen gain into the neutrophils is somehow restrained and only a small amount of H_2O_2 is produced by RB.

The controversial role of MPO

As described above, MPO seems to have a controversial role in phagolysosome killing. Still, it is the most abundant protein in neutrophils (23), and thus it is bound to have some importance in the vertebrate system. During infection and inflammation, there is also an abundance of MPO in the extracellular matrix and, it may have a role in extracellular killing. The extracellular pH is near 7, and at this pH the MPO-H_2O_2-halide system is still very actively functioning and producing HOCl or other hypohalide acids.

Discussion of programmed bacterial cell death

As discussed earlier and postulated also in the literature section, cell death through the genetically defined activation pathways also occurs in cells like *E. coli*, which possess characteristics of apoptosis, i.e. programmed cell death (PCD), (135-137, 139-141). This phenomenon is also present in the results section of this book with the manifestation of logarithmic degradation of the viability, especially in complement killing kinetics in Figure 19. We know that bacteria of different ages, or cells from cultures of different ages, differ in their susceptibility to the given agent. It has been reported that young cells are more readily destroyed than older ones (82, 135-138). The logarithmic dying pattern of a bacterial cell population can also be explained by this finding. The youngest population in patch culture is the largest one, and in the logarithmic death phase the first dying population is the largest one (Figure 7). This phenomenon can be dictated by the cytological resiliency of mature cells, forming more effective resistance than in the younger cell population. Triggering factors are partly the same as in sporulation and dormancy: disinfectants, heat, pressure, osmotic stress, DNA-damage, oxidative stress, the prevention of the phage infection and the prevention against the evasion of immune systems, like complement system and lysozyme (142) (II).
Still, not all killing possessed logarithmic killing kinetics, and this was the case when *E. coli*-lux was exposed to HOCl (figure represented in manuscript (VI) in original publications). In the MPO-H_2O_2-halide system, chlorination-derived killing is presumably caused by HOCl, produced by enzyme kinetic rates. When HOCl was added in the reaction as a bolus, the killing ceased to follow the logarithmic pattern. This is the topic of an on-going study, and the hypothesis is that the HOCl killing mechanism is a rapid oxidation of proteins with R–SH on the bacterial cell wall as the H_2O_2 induces a PCD reaction in the bacterial cell population.

5.4 Toxicological assessment with *E. coli*-lux (VIII)

The effects of ethanol and PmB were previously tested in order to reveal their basic antimicrobial effects against *E. coli*-lux. The investigation continued to get more detailed information on different antimicrobials and their killing effects. Could the *E. coli*-lux be used as a probe in assessing toxicity?

Susceptibility of E. coli-lux and sensitization

When bacterial cells are considered to be used as probe cells in toxicological assessments, it is important to clarify which mechanisms utilized by various cytotoxic agents and the mechanisms of single chemicals are outlined in the literature review section.
The first step was to study the effects of various organic solvents, like methanol and dimethyl sulfoxide (DMSO). *E. coli*-lux was found to be sensitive to both methanol and DMSO (data not shown), and concentrations higher than 5 % were refrained in assays.
Beta-lactam antibiotics like penicillin and its derivates, like ampicillin, did not affect the *E. coli*-lux due to the amp gene, producing the beta-lactamase enzyme providing resistance against these particular antibiotics (72, 175).

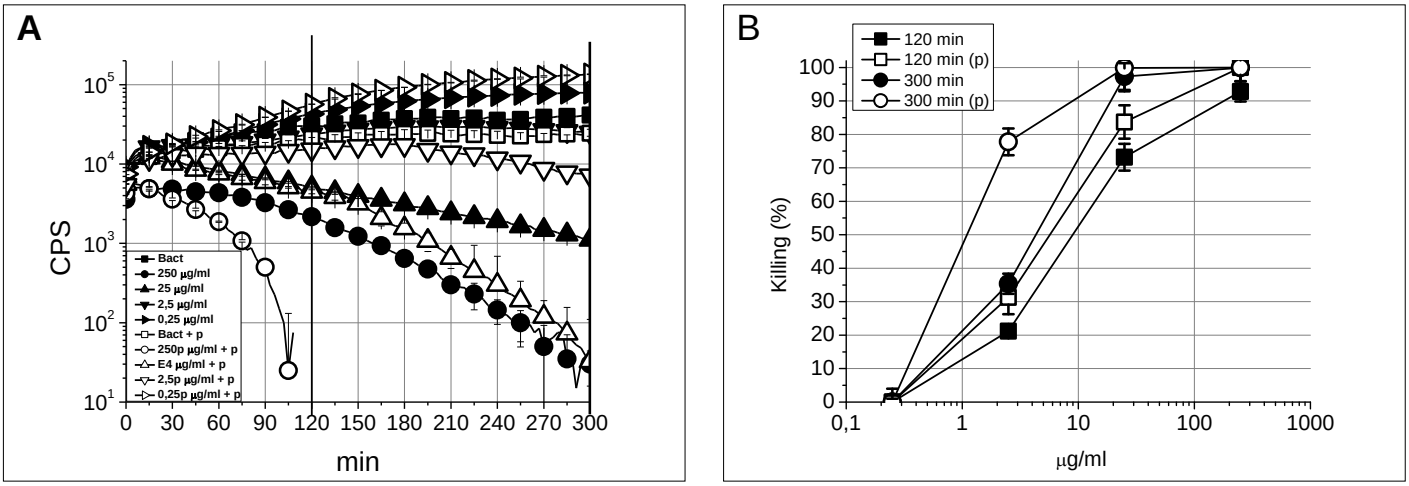


Figure 23: The bioluminescence (BL) kinetics of **(A)** *E. coli*-lux (10^5 cells) incubated in the presence of different concentrations [black symbols (square) 0 µg/ml, (triangle right) 0.25 µg/ml, (triangle down) 2.5 µg/ml, (triangle up) 25 µg/ml and (circle) 250 µg/ml] florfenicol in the plate reader in 100 µl HBSS at 37 C. All concentrations of florfenicol were also tested with 0.03 µg/ml polymyxin B (open symbols). **(B)** The dose-dependency of the florfenicol to the killing after 120 min (square) and 300 min (circle) of incubation. Open symbols represent the measurement in the presence of polymyxin B. The 100 % killing was defined as the CPS reading declined to the level of the background signal and the 0 % of killing as the CPS reading from the bacteria with no toxin added. CPS values are shown as the mean ±SD of measurements of three parallel wells.

E. coli is known to naturally resist some toxins by preventing their influx. For analytical purposes we can use sensitizers such as PmB. Acting like a detergent, PmB alters the permeability of the plasma membrane (177, 285). It is used in moderate concentrations, like 0.03 µg/ml, in the experiments shown in Figures 18 and 23, allowing intracellular transportation of the toxic agents without disrupting the cell.
Figure 23A represents the BL kinetics of the incubation of 10^5 *E. coli*-lux with florfenicol (antibiotic influencing ribosomes and protein synthesis, derived from chloramphenicol and used in farm animals

(141) (175)). Florfenicol samples and references were measured both with and without PmB, in order to fractionate between the possible intra- and extracellular toxicological effects. *E. coli*-lux was susceptible to florfenicol, and during the 300 min of incubation 250 μg/ml erased bacterial viability. When sensitized with PmB, already 25 μg/ml of florfenicol was enough to kill all the bacterial cells, and 250 μg/ml of florfenicol reduced viability to zero already after 100 min of incubation.
Figure 23B shows the dose-dependency at 120 min and 300 min of incubation, and Table 3 outlines the EC_{50} and EC_{90} values. PmB sensitized the *E. coli*-lux cells and enhanced and accelerated the impact of florfenicol.

Table 3: The effective concentration 50 % (EC_{50}) and 90 % (EC_{90}) of florfenicol after 120 min and 300 min of incubation, with (PmB) and without polymyxin B (0.03 μg/ml) from the measurements presented in Figure 23.

	EC_{50} (μg/ml)	EC_{50}^{PmB} (μg/ml)	EC_{90} (μg/ml)	EC_{90}^{PmB} (μg/ml)
120 min	9.3	5.7	90.1	60.8
300 min	4.4	1.1	19.5	8.5

Testing the antimicrobial effects of purified toxins

The antimicrobial effects of a wide range of different solvents, toxins and antibiotics were tested in a variety of different conditions in order to clarify the susceptibility of *E. coli*-lux to these agents.
Figure 24 represents the dose-dependency of six different toxins against 10^5 *E. coli*-lux cells after 180 min of incubation both with and without 0.03 μg/ml PmB. Deoxynivalenol (DON) (Vomitoxin) is a B trichothecene mycotoxin (Figure 24A) produced by the *Fusarium* mold commonly found in grains (189-195). Without sensitization, DON mainly manifested a weak bactericidal effect, and the EC_{50} value was reached with 1000 μg/ml. When sensitized with PmB, the probable intake of the toxin increased and the killing effect was notably enhanced with the EC_{50} value of 2.0 μg/ml, 500 folds lower than without PmB.
The same effect was present with moniliformin (MON) (Figure 24B), which is also a *Fusarium* toxin (189-195). EC_{50} of MON without PmB sensitization was 100 μg/ml and with PmB only 0.9 μg/ml. T-2-toxin (189-195), the third *Fusarium* toxin (Figure 24C), reported to be highly cytotoxic, had an EC_{50} value of 0.5 μg/ml, and when sensitized 0.04 μg/ml. With T-2-toxin and chloramphenicol (CHP) (Figure 24D), the broad-spectrum antibiotics, the PmB sensitization had an enhancing effect only in lower concentrations. The EC_{50} values for CHP were 0.4 μg/ml and 0.1 μg/ml with PmB.
Figures 24E and 24F present the dose-dependency of two antibiotics acting within the bacterial cell. Nalidixic acid is a quinolone derivate, disrupting DNA replication (141). After 180 min of incubation Nalidixic acid was not bactericidal but mainly bacteriostatic. EC_{50} value was 980 μg/ml and with PmB 300 μg/ml. Nalidixic acid is a DNA gyrase inhibitor, and this type of inhibition requires bacterial growth to be observed, and presumably this is a reason for the high EC values during 3h. Tetracycline is an antibiotic inhibiting the protein synthesis of a wide spectrum of bacteria. The EC_{50} values were nearly similar with and without PmB, the values being 46 μg/ml and 58 μg/ml respectively. This could result from the fact that the tetracycline molecule (146) can enter the plasma membrane quite efficiently even without any assistance or sensitization.
PmB is a practical tool for sensitization, but it must be noted that in organic solvents (like methanol or DMSO) it significantly increased the variation between the parallel measurements.

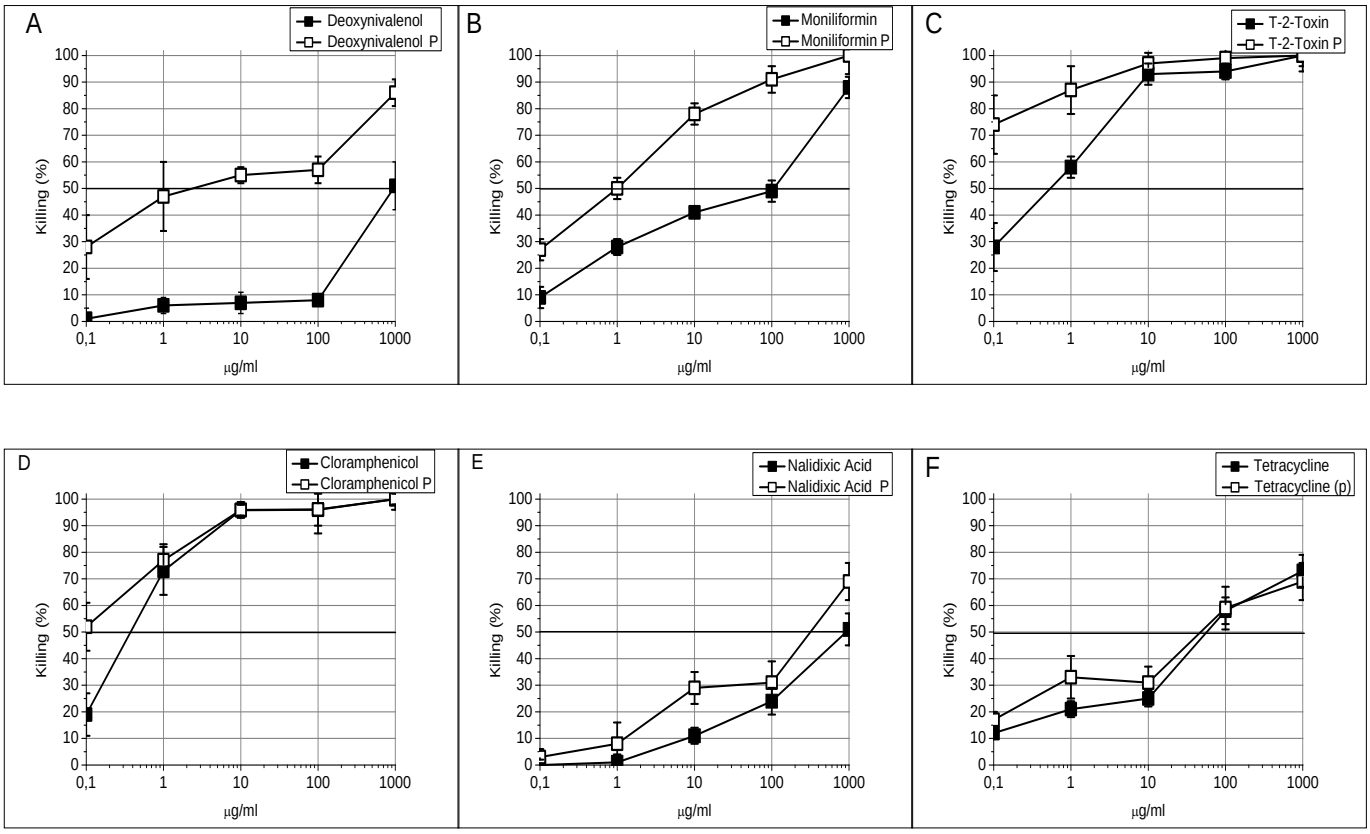


Figure 24: The dose-dependency of six different toxins after 180 min of incubation **(A)** deoxynivalenol, **(B)** moniliformin, **(C)** T-2-Toxin, **(D)** Chloramphenicol, **(E)** Nalidixic acid and **(F)** Tetracycline against 10^5 *E. coli*-lux cells after 180 min of incubation in 100 μl HBSS at 37 C both with (open symbols) and without (black symbols) 0.03 μg/ml PmB. The 100 % killing was defined as the bioluminescence reading declined to the level of the background signal and the 0 % of the killing as the CPS signal from the bacteria with no toxin added.

Testing the extracts from toxic molds (VIII).

Normally, toxicity is a combination of numerous complex factors and synergism (360, 361). Previously, we showed the very simplest example of synergism, with two factors, when PmB was used as a sensitizer with toxins and antibiotics (Figures 23 and 24). The measurement of total toxicity was revealed when extracts from 6 species of indoor molds [*Aspergillus versicolor* (Av), *Aspergillus westerdijkiae* (Aw), *Paecilomyces variotii* (Pv), *Penicillium expansum* (Pe), *Trichoderma atroviride* (Ta) and non-toxic reference *Trichoderma reesei* (Tr)], collected from mold-damaged houses and pure laboratory cultured (Food and Environmental Sciences, Helsinki University, Helsinki, Finland), were studied (Figure 25). As a reference, the same extracts were exposed to boar sperm cells and PK-15 porcine kidney epithelial cells and their viability was assessed (VIII). These methods are commonly used in cytotoxicity measurements (184, 186, 362-364).

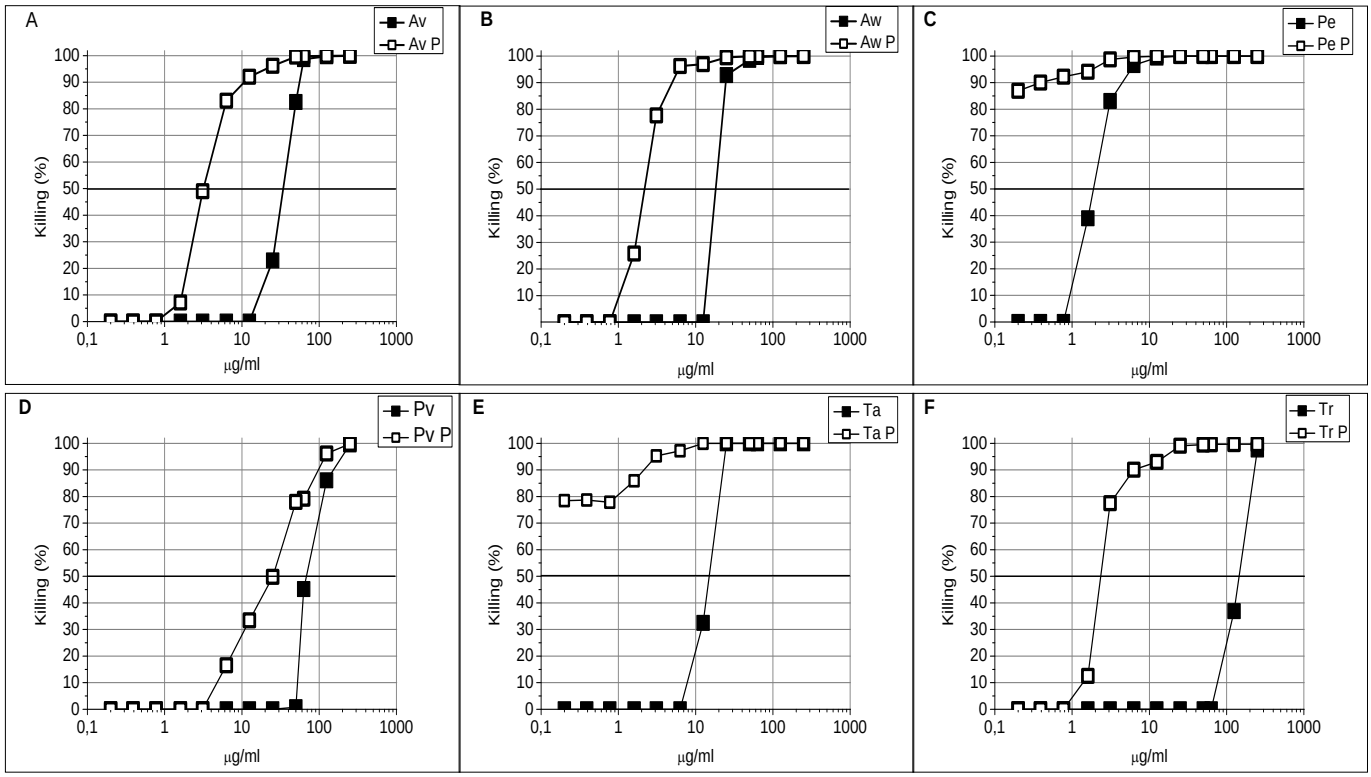


Figure 25: The dose-dependency of extracts from 6 molds after 240 min of incubation **(A)** *Aspergillus versicolor,* **(B)** Aspergillus westerdijkiae, **(C)** *Penicillium expansum*, **(D)** *Paecilomyces variotii*, **(E)** *Trichoderma atroviride* and non-toxic reference **(F)** *Trichoderma reesei* against 10^5 *E. coli*-lux cells in 100 µl HBSS at 37 C both with (open symbols) and without (black symbols) 0.03 µg/ml PmB. The 100 % killing was defined as the CPS reading declined to the level of the background signal and the 0 % of the killing as the CPS reading from the bacteria with no extract added.

Results indicated that similar doses of metabolites from mold extracts sufficient to decrease *E. coli*-lux viability were also inhibiting the viability functions of PK 15 cells and the motility of boar sperm cells (VIII). *E. coli*-lux was less susceptible to the extract from Tr (Figure 25F) selected for non-toxic reference. It was selected as a reference sample because the extract did not inhibit PK-15 cell viability or boar sperm motility. All three cells seemed to be very susceptible to the extract from Pe (VIII).

Sensitization with PmB made *E. coli*-lux more susceptible to all extracts (Figure 25), and the reference sample also became toxic (Figure 25F). The results of *E. coli*-lux and boar sperm cells are outlined in the Table 4.

Table 4: The effective concentration 50 % (EC_{50}) and 90 % (EC_{90}) of extracts from 6 molds [*Aspergillus versicolor* (Av), *Aspergillus westerdijkiae* (Aw), *Paecilomyces variotii* (Pv), *Penicillium expansum* (Pe), *Trichoderma atroviride* (Ta) and non-toxic reference *Trichoderma reesei* (Tr)] against boar sperm cells and 10^5 *E. coli*-lux (VIII).

Molds	Sperm Cells		*E. coli*-lux			
	The inhibition of mobility		The reduction of bioluminescence			
					Polymyxin B (0.3 µg /ml)	
	EC_{50} (µg/ml)	EC_{50} (µg/ml)	EC_{90} (µg/ml)	EC_{50} (µg/ml)	EC_{90}, (µg/ml)	EC_{50} (µg/ml)
	1 h	24 h	4 h	4h	4 h	4h
Av	250	10	50	34	10	3
Aw	10	5	20	18	4	2
Pe	10	1	4	2	0,4	<0.1
Pv	25	5	130	69	90	25
Ta	10	3	15	12	2	<0.1
Tr	250	>50	200	145	6	3

5.4.1 Toxicity analysis from indoor dust

The toxicity testing application for indoor air assessment includes both the sample collection kit and analysis system with *E. coli*-lux cells as an inductor. The test system reveals the "total toxicity" of the sample, taking into account the toxicity from all sources and not just the microbe-derived toxicity. Indoor dust was collected from at least three points of a room to exclude possible "background noise" caused by detergents and other factors cumulated in normal indoor dust. From each detection point two samples were swiped using microfibre swipes (in total 6 samples/ room) (Figure 26). Dust was weighted and suspended both in H_2O and in DMSO in 10 mg dust/ml. The extract was diluted into the desired concentration and subjected to *E. coli*-lux. Results were plotted in the dose-response curve showed in Figure 27.

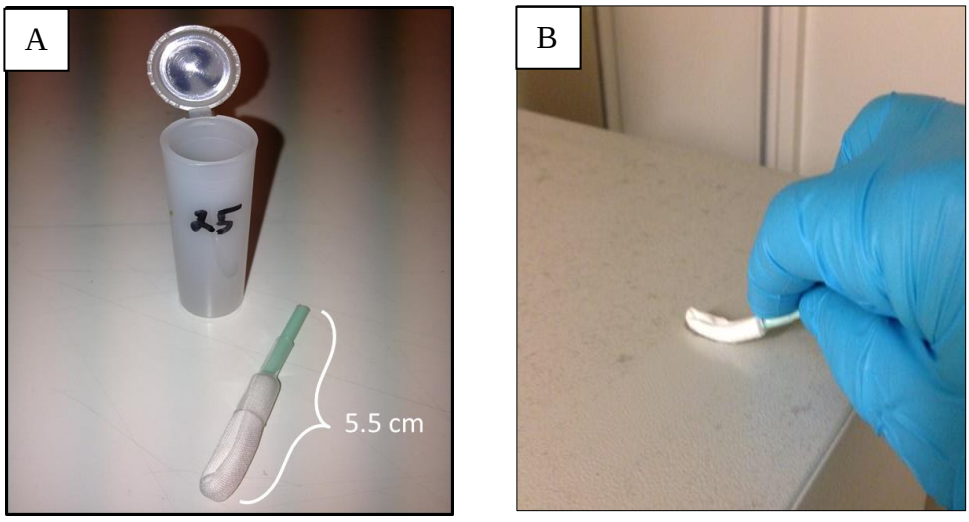


Figure 26: (A) The indoor dust sampling kit, the swipe and the tube, innovated during the project. Before sampling, the tubes containing the swipes are weighed. **(B)** Indoor dust sampling in progress. After sampling, the tube with the swipe is reweighed in order to gain the quantity of the collected dust.

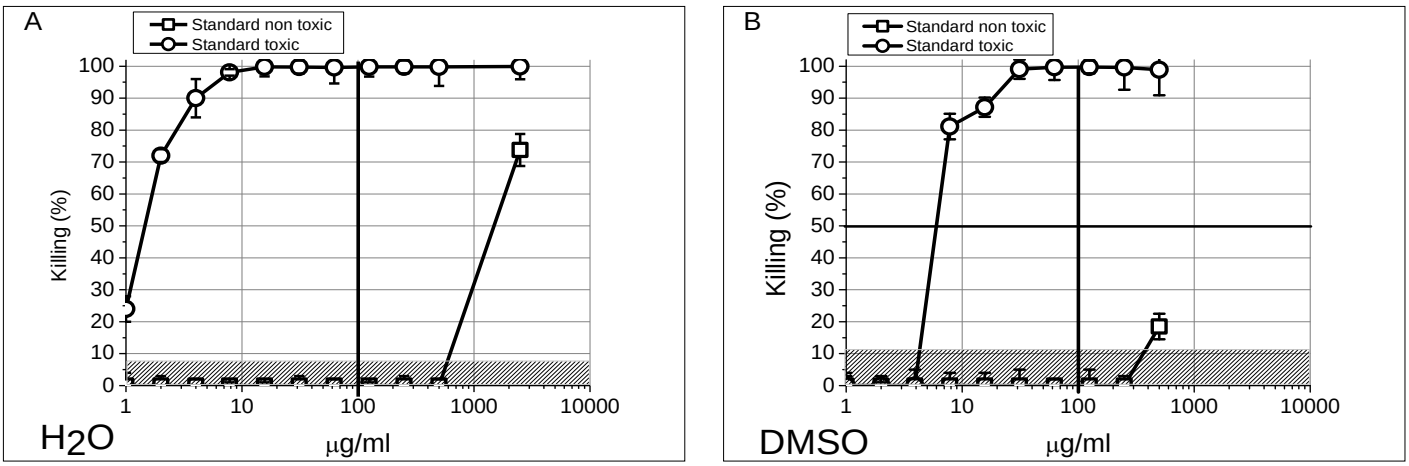


Figure 27: The results of the standard dust samples diluted in H_2O **(A)** and DMSO **(B)** taken from the known targets. Open squares represent the 120 min dose-dependency against *E. coli*-lux (10^5) cells of the non-toxic (square) and the toxic sample (circle). The grey bar represents the "signal to noise ratio" (9.2 % in H_2O and 11.7 % in DMSO dilutions). The 100 % killing was defined as the CPS reading declined to the level of the background signal and the 0 % of the killing as the CPS reading from the bacteria with no toxin added.

Figure 27 represents the standard dust samples collected from the targets known to be either highly toxic or non-toxic interiors. The grey area represents the signal to noise ratio of the measurement system, which was on average 9.2(±1.4) % in H_2O and 11.7(±1.7) % in DMSO dilutions. The signal to noise ratio was determined as a portion of the standard deviation (3 parallel measurements) from the BL signal without the toxic sample. The testing of 951 samples from 65 different, damaged and non-damaged, buildings (14 public and 51 private) let us outline the toxic threshold values.
The limit value of the toxic sample was determined to be EC_{50} = 100 µg/ml, both in H_2O and DMSO dilutions, and the results were divided into four categories, which are summarized in Table 5. The third class is an ambiguous category needing more detailed knowledge on the target. This class is usually considered to be non-toxic, but if for example all the other samples from the target are highly toxic we can also interpret this one to be as well, or vice versa.

Table 5: The toxicity categories used. Classes I and II are clearly toxic, III is an ambiguous category and IV clearly non-toxic.

Class	EC_{50}	Toxicity
I	EC_{50} < 25 µg/ml	Highly Toxic
II	EC_{50} = 25 – 100 µg/ml	Toxic
III	EC_{50} = 100 – 250 µg/ml	Compromised
IV	EC_{50} > 250 µg/ml	Non-toxic

Figure 28 represents four categories in practice, representing four samples of one mold-damaged school building in Southern Finland. From this building, we diagnozed all the four different toxicological classes. Our application proved to have a good ability to differentiate and fractionate the toxic areas inside the damaged buildings. PmB sensitization was not utilized in these experiments, represented in Figures 27 or 28, because its additional value for the results was determined to be low.

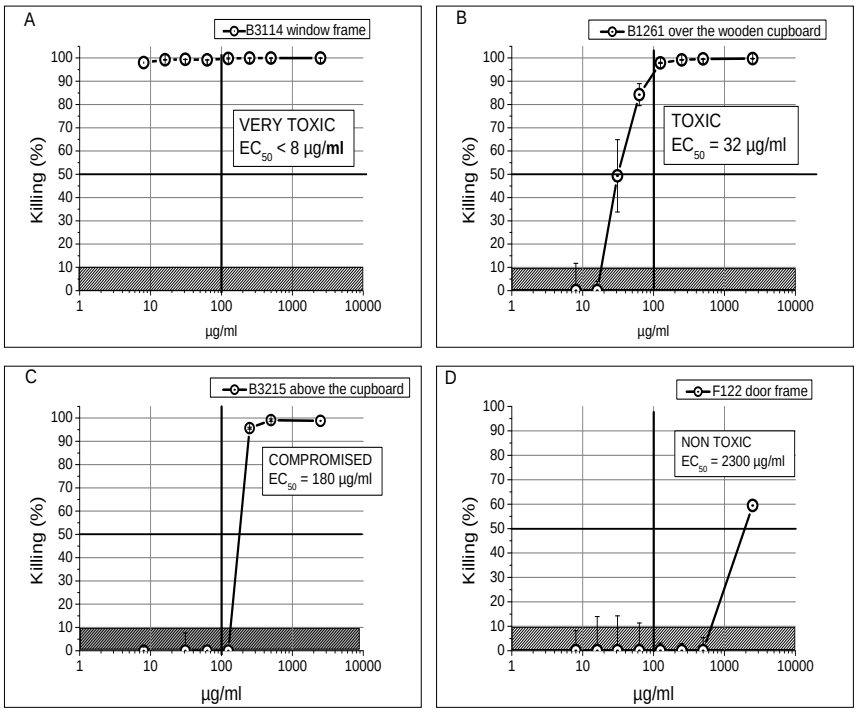


Figure 28: The results of the dust samples collected from different spots of the mold-damaged school building in Southern Finland. Circles represent the 120 min dose-dependency of the sample dilutions (H_2O) against *E. coli*-lux (10^5 cells). The grey bar represents the signal to noise ratio of the measurement system. The 100 % killing was defined as the CPS reading declined to the level of the background signal and the 0 % of the killing as the CPS reading from the bacteria with no toxin added. All four toxicity classes (table 5) were discovered from the results in the current study.

In the TOXTEST project (part of the Hometalkoot project funded by the Ministry of Health and Welfare 2010-2012) (365), indoor dust samples were collected from 11 damaged and 11 healthy targets. Target buildings were diligently chosen on clinical and constructional bases by medical doctors and construction engineers, and both the occupants and the buildings were thoroughly diagnosed. Toxic analysis included, inter alia, *E. coli*-lux and boar sperm cell applications. Despite the diligent preparation, the sampling from the targets was unsuccessful and the samples were not representative (365). *E. coli*-lux application recognized the damaged buildings in 80 % and 60 % specificity and sensitivity, respectively, when just dust samples and the targets were compared (365). The TOXTEST data was reanalyzed in 2014 in cooperation with Professor M. Salkinoja-Salonen (Food and Environmental Sciences, University of Helsinki, Helsinki, Finland) and by Professor M. Gasik (Aalto University, Espoo, Finland), and the sensitivity and specificity combined with the boar sperm cell test results were >75% and 100 % respectively when compared with the health status of the buildings and their users (Figure 29).

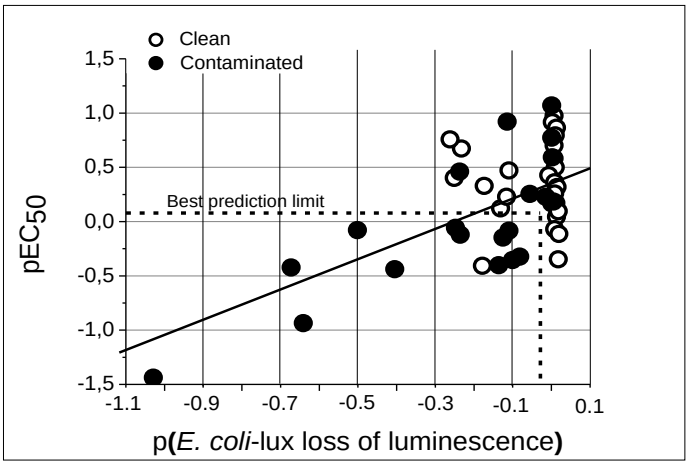


Figure 29: The Bayesian analysis (366) allowed the selection of the threshold values for ill-health symptoms allowing high sensitivity (>75%) and specificity (nearly 100%) values to classify the toxic status of the indoor space by the *E. coli*-lux application. EC_{50} represents the lowest concentration of dust substance that was cytotoxic to 50 % of exposed sperm cells compared with vehicle control. Remarkably toxic samples were found in 11 sites. Data is expressed as pEC50 ($-\log_{10}[25/EC50]$) correlated with the loss of luminescence of *E. coli*-lux. Clean buildings (open circle) and contaminated (black circle)

5.5 Toxicological assessment using neutrophils (VI)

Neutrophils are good indicators of the pathophysiological state of the individual (335-337). As described earlier, the luminol amplified CL method is directly linked to neutrophil activity. Neutrophils can be used as a probe for toxicological assessments either directly exposed to the toxins or measuring PMN activity of the exposed individuals (V, VII).

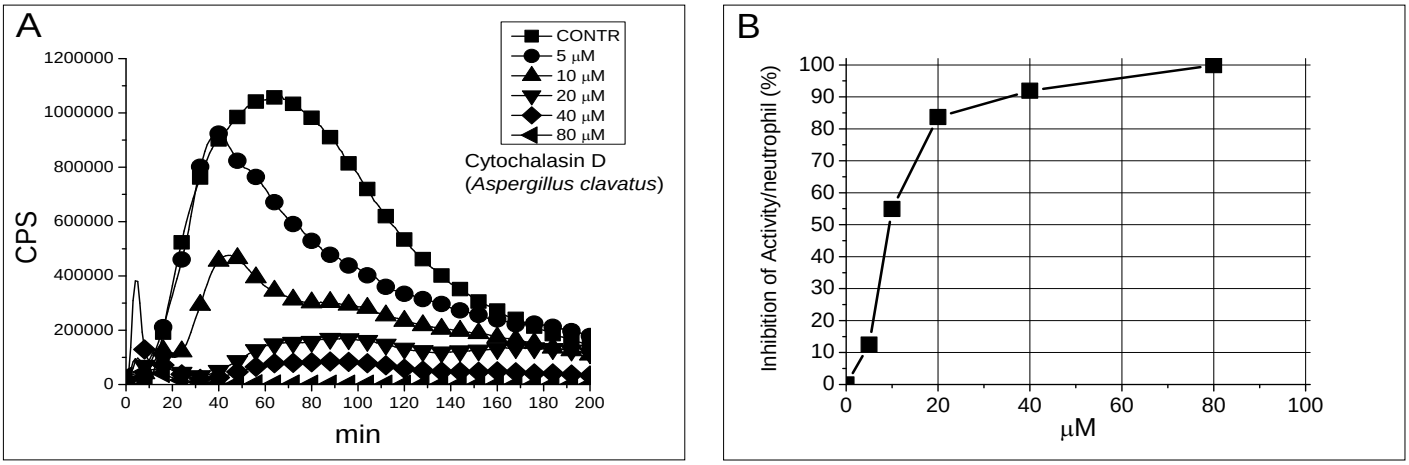


Figure 30: (A) the effects of the cytochalasin D 0 µM (■), 5 µM (●), 10 µM (▲), 20 µM (▼), 40 µM (◆) and 80 µM (◀) to the luminol aplified chemiluminescence signal of human neutrophils, induced by the zymosan in 200 µl HBSS at 37 C. Dose-dependency is presented in **(B)** as an inhibition related to the maximum emission peak of the reference measurement without the toxin (0 % inhibition). The effects of the toxin on the activity/cell, i.e. the peak of the CL emission per neutrophil, takes into account the number of used neutrophil cells and is designated as specific toxicity (■).

Cytochalasin D decreased the luminol amplified CL peak value of the human neutrophils activated by the zymosan (Figure 30A). The response is presented in Figure 30B as related to the maximum emission peak of the reference measurement without the toxin (**VI**). The effects of the toxin on the activity/cell, i.e. the peak of the CL emission per neutrophil, is shown in Figure 31B, which takes into account the number of used neutrophil cells and is designated as specific toxicity (VII). The EC_{50} for cytochalasin D was 9 µM.

Figure 31 presents the response of mycotoxins. These same toxins were assessed with the *E. coli*-lux system using the same mycotoxin concentrations represented in Figure 24.

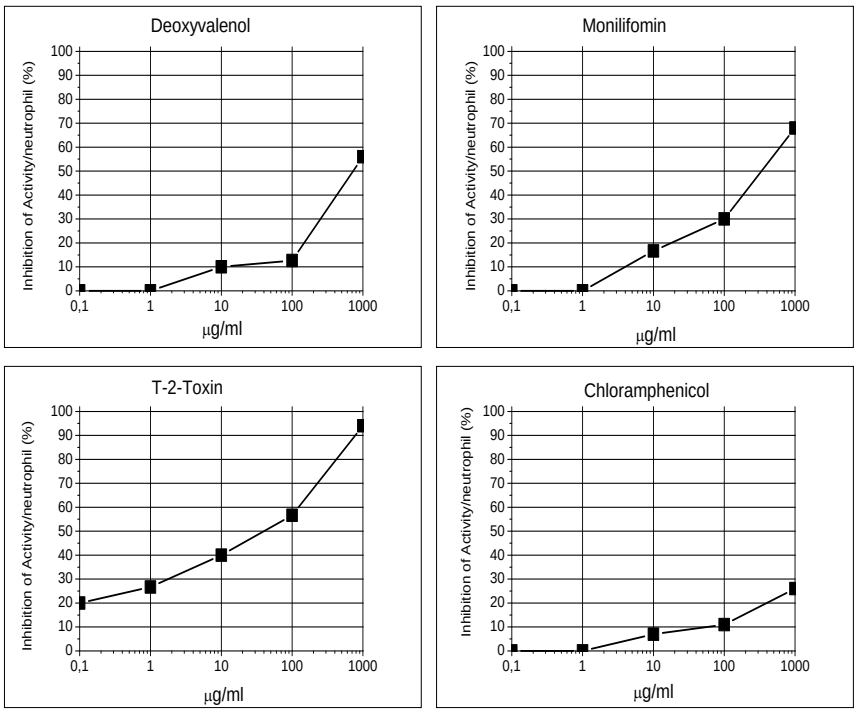


Figure 31: The specific toxicity of different toxins **(A)** deoxynivalenol, **(B)** moniliformin, **(C)** T-2-Toxin, **(D)** Chloramphenicol, to the activity/neutrophil. Maximum emission peak of the reference measurement without the toxin was set as a 0 % of inhibition.

Neutrophils can be used as a probe in the toxic assessment of different chemicals. The application is restricted to H_2O soluble agents, as neutrophils are sensitive and susceptible to organic solvents such as methanol and DMSO.
Nevertheless, CL analysis is a practical method for testing the effects of different toxins on mammalian cells, and moreover, analysis can be done directly from whole blood dilutions without cell separation. The required amount of blood is very low, enabling fingertip sampling and the usage of small test animals like rats. Luminol derived CL is linked to the release of MPO from the azurophilic granules of the phagocytes (342). It has been reported that in whole blood the major CL emission comes from neutrophil activity; the alternative CL source, the monocytes, emits only about 50 % of neutrophil emission, and neutrophils represent nearly 90 % of the whole phagocytic leukocyte population (342, 347).
The CL emission method was utilized when MON was administered to rats in a subacute toxicity test orally on a daily basis for 28 days (VII). Rats were divided into 8 groups on account of dosage, including one control group without MON. MON, even in the smallest dosage, significantly decreased neutrophil activity compared with that of a control group, indicating an adverse effect on innate immunity (VII).

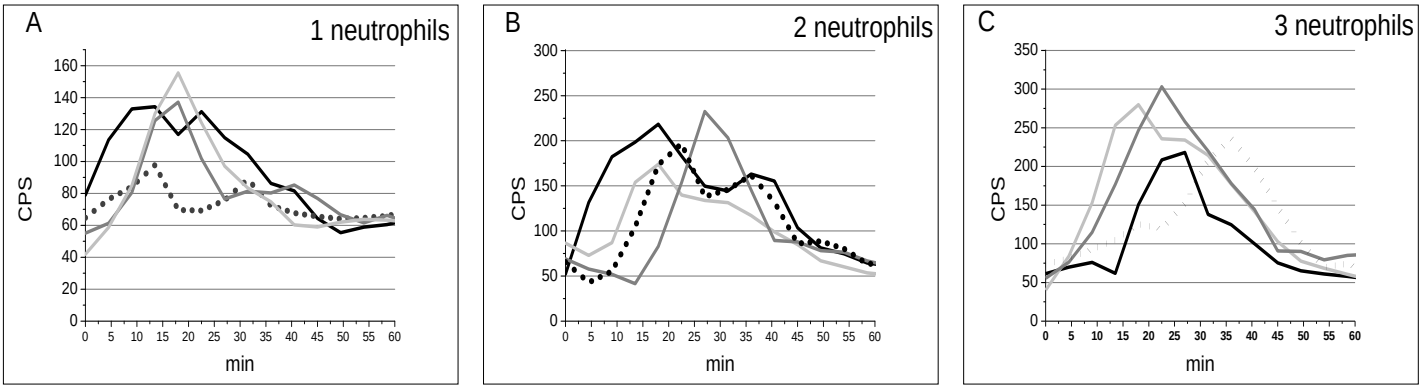


Figure 32: The chemiluminescence signals of four parallel dilutions containing **(A)** 1, **(B)** 2, or **(C)** 3 neutrophils from a single donor.

Figure 32 shows unpublished data from an experiment setting where cell suspension was diluted until the background signal was reached and signals diverging significantly from the background (background + 3SD) were observed. Figure 32 shows the CL responses of various dilutions containing the hypothetical number of neutrophils. The wells with 1, 2, or 3 neutrophils (four parallel dilutions for each cell number) gave peak CL responses of 139(±27) CPS, 226(±28) CPS, or 300(±25) CPS, respectively. Thus, the peak CL value of one neutrophil was 81(±2) CPS. The mean peak CL value for one cell from 4 different donors was 70(±9) CPS. The peak time of a single cell for one donor varied from 10 to 30 min. A similar analysis was also done from whole blood dilutions with similar results (data not shown). When the peak CL responses of the neutrophils of four different donors were plotted against the cell number, linear correlation was obtained (data not shown). The single cell peak responses were remarkably constant, revealing little heterogeneity. The peak time, on the other hand, varied between 10 and 40 minutes and the onset of the reaction between 0 and 20 min.

The single cell method provides a potential technique for the biomonitoring of the users of the toxic buildings alongside toxicity analysis.

6. CONCLUSIONS AND FUTURE PROSPECTS

6.1 Insight into the serum complement system and neutrophil activity

E. coli-lux application is especially suitable for the assessment of innate immunity activity, namely the activity of the serum complement pathways and the phagocytosis (I, II, III, IV, V, VI). In the future, regarding the complement system, component depleted or deficient serum samples are used to clarify the true impact of the individual components and separate pathways on microbe killing. Simultaneously, flow cytometer can be utilized to find out the composition of the complement proteins and proteases attached to the bacterial surface at a given time of the reaction. The same procedures can be extended to phagocytosis assessment when bacteria in different phases of "opsonization" are targeted at neutrophils, and bacterial killing efficiency is recorded, combined with luminol and lucigenin amplified chemiluminescence (CL) measurements.
Killing by neutrophils, especially concerning the entity of the phagocytosis, is still under incessant investigation. To conclude, we showed that the phagolysosomal concentration of H_2O_2 is high enough (pH 7.8) to support the killing of one bacterial cell without MPO participation (VI). The chlorination activity of the MPO was drastically reduced at a higher pH.
The number of bacteria in the reaction mixture has a large impact on the concentrations of agents needed for the killing. This is why the testing of the killing parameters with the variable concentrations of MPO-H_2O_2 system utilizing different halides and halide concentrations at different pH will provide an adequate research project reaching far into the future.

6.2 Microbiological and biochemical sentiments

With the advantages of BL measurement, regarding the real-timeness and easy-to-do assaying, it is a considerable option for viability and killing assessments. Plate counting viability assay, the golden standard in microbiology, is a quite tedious and time-consuming method, and for example the kinetical measurement requires a preparation of huge numbers of plates. Moreover, plating completely lacks real-time properties, since the results are obtained after long incubation periods. We also showed that there is a bias in the long incubation period, since in the antimicrobial assays the antimicrobial agent usually follows into the culture agar, continuing the reaction during the overnight incubation, and thus affecting the results. This was visible especially in our complement and in neutrophil/MPO-H_2O_2-halide assays as advanced killing on the plates compared with the real-time data (II, VI).
E. coli-lux also possesses the genes producing enhanced green fluorescent protein (EGFP) (II). The gene is located under the same powerful lac-promoter as the lux-gene-cassette and produces EGFP protein. EGFP is a good detector of the cell number, and measurable with the plate reader, but as a highly stabile protein it stays actively fluorescent after the bacteria had died and even lysed. Thus it is not practical for *E. coli*-lux killing assessments and has not been used in these studies. The plate reader also measures optical density (OD) in real-time, but the sensitivity was low and the measurement dynamics not comparable with the BL application (II). This disadvantage was however improved in the newer models of the plate readers.
The ideal setting in microbial assessments would be measuring three parameters simultaneously, the BL signal representing viability and killing, OD the growth and lysis and the fluorescence from the EGFP the estimation of the number of bacterial cells.
The BL signal correlates directly with the number of bacterial cells in the reaction, and in Hidex plate readers this ratio is close to 0.1 CPS/CFU (II). The same principle and ratio apply with the minor aberrations in bacterial killing. The diminishment of the BL correlates with the number of killed bacterial cells. The application is capable of assessing reactions containing 10^2 to 10^9 bacterial

cells, and the plate reader utilizing microtiter plates with 96 wells provides a nearly high-throughput application for the analysis. The BL application of *E. coli*-lux is a practical and advantageous method for viability assessment.
The restriction of the application is that it requires a gene-manipulated organism, like *E. coli*-lux. Our consideration in the future is to utilize another, gram (+) organism, like *S. aureus*, transformed with the same construct to be used especially in immunological studies, mainly in the complement system and phagocytosis.

6.3 Toxicological assessment

E. coli-lux, constitutively emitting bioluminescence (BL), was developed and utilized as a convenient tool for the analysis of the antimicrobial properties of different antimicrobial agents. It was progressively used to detect the kinetical features of the serum complement system, neutrophil activity and the toxicity of different chemicals and factors (I, II, III, IV, V, VI, VII, VIII).
There is a generalized opposition against the use of bacterial cells as toxicological probes because of the assumption that bacterial cells would not be susceptible to many toxins. Nevertheless, we have shown that *E. coli*-lux is actually very handy and cost-effective for just these purposes, and moreover, exactly comparable and parallel with the conventional mammalian cell probes (VIII). The handicap of the *E. coli*-lux application is its resistance to beta-lactam antibiotics. It is known that some species of fungi may produce these or related molecules which are thus not detectable with the current *E. coli*-lux method. Still, the sensitivity and the specificity combined with the boar sperm cell test results were >75% and 100 % respectively, when compared to the health status of the buildings and their users.
The analysis and sampling kit for the indoor air total toxicity testing (not just the microbe derived toxicity) is underway to be completed, as the application will be officially accredited in 2015.
Alongside the *E. coli*-lux, the neutrophils with the CL emission ability were used as a reference method, revealing the cytotoxic effect against mammalian cells. When rats were subjected to moniliformin, the neutrophil activity was assessed to decrease significantly during the 28 days of the exposure (VII).
In addition to the cytotoxic characters of the toxins, the biomonitoring of the users of the damaged buildings is also a part of the on-going project. Like on the neutrophils of the rats previously, the effects of the toxicity on the neutrophils of the building users will be tested by assessing the luminol amplified CL emission of the fingertip whole blood samples. As presented in the results, we can measure the CL emission of one neutrophilic cell, and this one-cell-emission technique might appear to be especially applicable for biomonitoring purposes.

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