



## Microbes, molecules, maladies and man

Adriano G Duse

*Dedication:* To Professor Hendrik J Koornhof, mentor, friend and man with immense knowledge, insight, wisdom and compassion, who has been a true inspiration to all those who have had the privilege to know him.

The planet Earth was formed, in a molten state, some 4.5 billion years ago. It cooled off sufficiently 4 billion years ago to allow the formation of the oceans. Cyanobacteria, found in rock fossil records that are approximately 3.6 billion years old, provide the earliest evidence of life in the form of complex unicellular organisms. Molecular phylogeny is the tool that enables us to understand life in all its complexity and recognise relationships between organisms. In essence, using molecular techniques, we are able to determine the evolutionary relationships of living creatures. By comparing the difference in sequences of homologous genes encoding ribosomal RNA (16S rRNA genes from prokaryotic cells and 18S rRNA from eukaryotic cells) we can measure the evolutionary distance between species of organisms. Computer analysis of rRNA gene sequences suggests that cellular life has evolved along three major lineages. Two of these, Bacteria and Archaea, are exclusively microbial and consist of prokaryotic cells. The third lineage, Eukarya, not only contains unicellular organisms but also myriad multicellular organisms. Two important points have emerged from the study of molecular phylogeny: (i) unicellular organisms are the major and most diverse form of life; and (ii) eukaryotes are not of recent origin, as previously thought, but as ancient as the Bacteria and Archaea lineages, all of which have emerged from a universal ancestor. Although the human race may live in harmony, and is subject to colonisation with many different prokaryotic (e.g. bacteria) and eukaryotic (e.g. fungi and parasites) organisms, this harmony is shattered from time to time when a relatively restricted number of microbial species enter our body and cause pathology-infection.

Antimicrobials, developed on a mass scale from the 1940s onwards, brought renewed hope that, to quote the UN Surgeon General in his address to Congress in 1969, 'We can close the book on infectious diseases'. Unfortunately, nothing could have been further from the truth. With the continuous emergence of microbes that display a vast array of antimicrobial resistance

*Chief Specialist and Academic Head, Department of Clinical Microbiology and Infectious Diseases, School of Pathology, National Health Laboratory Service, and University of the Witwatersrand, Johannesburg*

**Professor Adriano G Duse**, MT, MB BCH, DTM&H, MMed (Microbiol), FCPATH (SA) (Microbiol)

*Corresponding author: A G Duse (agaduse@icon.co.za)*

mechanisms, the 'golden era' of antimicrobials is under serious threat. Not only has mankind been challenged by multiple drug-resistant (MDR) pathogens, but the disturbing re-emergence of infections thought to have been controlled or defeated (e.g. tuberculosis) together with the emergence of relatively new infectious agents (e.g. *Clostridium difficile*, Ebola virus, prions) and hitherto unknown organisms (e.g. SARS-coronavirus and metapneumovirus) is evidence that mankind is continually challenged by infectious entities. By the end of the 20th century, it was estimated that, globally, 50 million people die each year; of these, 20 million die as a result of infectious diseases.

The aims of this article are to:

- Understand the concept of infectious disease causation
- Elucidate host-parasite interactions and discuss the genesis of infectious diseases
- Understand the complexity of bacterial interactions within biofilms
- Appreciate the contribution of the molecular biology era in enabling us to understand and diagnose the causative agents of infectious diseases.

### Causation of infection

The beginning of the search for microbial causes of infection was during the 1840s when a German anatomist Friedrich Henle, first hypothesised the existence of infectious agents that may have caused certain infectious diseases. Although Henle accepted that microbes (pathogens) could cause disease (symptomatic infection), he emphasised the fact that causation of infection by a pathogen with disease (infection) could not be simply assumed by the association of infection with a microbe. In 1840, as quoted by Fredricks and Relman,<sup>1</sup> Henle wrote: 'One could prove empirically that organisms were really effective only if one could isolate ... the contagious organisms from the contagious fluids (from the infected host), and then observe the power of each separately'. While Henle pursued his academic endeavours at the University of Gottingen, Robert Koch enrolled at this Institution in 1862. It is not clear to what extent Henle's ideas influenced Koch on the subsequent development of his postulates on how to prove the causal relationship between an infectious agent and its associated disease. The Koch's postulates, as they have become known, were presented in 1890 at the 10th International Congress of Medicine in Berlin and state the following:<sup>2</sup> (i) the micro-organism occurs in every case of infection under circumstances that account for both the pathological features and the clinical



course of the infection, (ii) the micro-organism does not occur in any other disease as a fortuitous and non-pathogenic agent, (iii) after being cultured from the diseased host and repeatedly grown in pure, axenic (artificial, lifeless) culture, the micro-organism causes the same disease in a new host, and (iv) the micro-organism must be recovered once again from pathological lesions of this new host. The fourth requirement was subsequently added to the Koch's postulates.

The key concepts in the Koch's postulates are: (i) specificity of the association of a micro-organism with an infectious condition; (ii) biologically and scientifically plausible correlation of microbiological, pathological and clinical features; (iii) isolation of the causative micro-organism by culturing it on laboratory media; and (iv) reproduction of disease by inoculating the isolated micro-organism into a susceptible host. The aim of the Koch's postulates was, by virtue of the criteria contained therein, to determine the association between micro-organisms and infection thereby formalising the connection between cause and effect in clinical medicine.

Certain limitations, pointed out by Fredricks and Relman,<sup>1</sup> inherent in the Koch's postulates were already evident in the 1800s. Although, at the time of Koch, *Vibrio cholerae* was recovered from patients with cholera, it was also isolated from healthy individuals, thus defying Koch's second postulate that stipulates the importance of the specificity of association of microbe with disease. With our increased understanding and elucidation of causation of infectious diseases, the limitations posed by the original Koch's postulates remain frustrating. Scientists are still struggling with laboratory culturing of *Mycobacterium leprae* – the cause of leprosy. Not being able to adequately isolate *M. leprae* in pure cultures impedes the fulfilment of Koch's third postulate. Other examples in which one or more of the Koch's postulates are not fulfilled include:<sup>1</sup> (i) organisms such as *Plasmodium falciparum* and herpes simplex virus or other viruses cannot be grown in cell-free, lifeless cultures, yet their pathogenicity is unequivocal; (ii) organisms that do not cause disease in carriers (e.g. *Neisseria meningitidis*) or those causing sub-clinical infection (e.g. *Mycobacterium tuberculosis*); (iii) organisms capable of toxin production causing distant injury from the infecting site or autoimmune reactions; (iv) organisms requiring co-infection with bacteriophages, e.g. *Corynebacterium diphtheriae* to acquire genes that encode for toxins that cause clinical disease; (v) viruses that require co-infection with another virus before causing infection, e.g. hepatitis D with B; (vi) organisms such as HIV that cannot produce typical disease in other hosts; and (vii) other host (including genetic) and environmental factors that are not taken into consideration.

Koch's postulates were used extensively and successfully as an experimental methodology to describe many microbes and the diseases they cause in medical microbiology. However, these criteria did break down and had to be modified with the discovery of viruses and the birth of virology that occurred

during 1886 - 1903. Viruses, as we understand them today are at the threshold of life in their design and function. They are non-cellular packages of protein and genetic material lacking any physiological material of their own thus making them obligate intracellular parasites and completely dependent on a host for survival. The emergence of virology, as a science, led to the need to accommodate the problem of proving disease causation by viruses. Rivers, in 1937, proposed his own postulates:<sup>1,3</sup> (i) a virus associated with a disease should be present with a high degree of regularity; and (ii) the virus associated with an ill, infected, host must not be a fortuitous finding but be the actual cause of disease. Rivers made provision for the concept of a viral carrier state with the abandonment of the need to grow pathogenic viruses in media or cell culture. In addition, in order to establish the link between a virus and a specific disease, the pathogenic virus should be associated with specific pathological lesions during infection. Inoculation of infected material into susceptible hosts should also produce disease, and/or the production of specific antiviral antibodies, with some regularity in comparison to appropriate controls.

In 1957, Huebner proposed the introduction of immunological criteria, such as the development of specific antibodies to epitopes on an infectious agent or the prevention of the infection by a specific vaccine and, in addition, emphasised the importance of taking epidemiological criteria into account when attempting to determine the relationship between viruses and disease.<sup>1,4</sup> Hill in 1965 eloquently described a number of epidemiological criteria that could be useful, in conjunction with the many revisions of the Koch's postulates, to distinguish between disease causation and association.<sup>5</sup>

A further set of criteria for causation was developed by Evans in 1976.<sup>6,7</sup> These were based on information that could be obtained using modern techniques, a greater understanding of the pathogenesis host-microbe interactions, and an insightful recognition of the limitations of the original Koch's postulates. The Evans criteria, as listed by Fredricks and Relman,<sup>1</sup> are:

1. The prevalence of a disease should be significantly higher in those exposed to the putative cause than in control cases where there is no exposure.
2. Exposure to the putative cause should present more commonly in those with disease than in controls without the diseases when all risk factors are held constant.
3. Incidence of the disease should be significantly higher in those exposed to the putative cause than in those not so exposed, as shown in prospective studies.
4. Temporally, the disease should follow exposure to the putative agent with a distribution of incubation periods on a bell-shaped curve.
5. A spectrum of host responses should follow exposure to the putative agent along a biological gradient from mild to severe.



6. A measurable host response following exposure to the putative cause should regularly appear in those lacking this exposure or should increase in magnitude if present before the exposure.

7. Experimental reproduction of the disease should occur in higher incidence in animals or humans appropriately exposed to the putative cause than those not so exposed; this exposure may be deliberate in volunteers, experimentally induced in the laboratory, or demonstrated in a controlled regulation of natural exposure.

8. Elimination or modification of the putative cause or of the vector carrying it should decrease the incidence of disease.

9. Prevention or modification of the host's immune response on exposure to the putative cause should decrease or eliminate the disease, and

10. It should make biological and epidemiological sense.

The study of emerging or new viral diseases is complex and includes interpretation of the interaction between three important factors: the environment, the microbe and the host. Once epidemiological and serological criteria have been met, new viral diseases are further described using classic laboratory methods of virus isolation, culture in cell-containing media, and sequence-based molecular techniques. Using these various epidemiological criteria and laboratory techniques, many new viruses have been described since the 1970s including Marburg virus in 1976, Ebola virus in 1977, and chronic viral diseases like hepatitis C in 1989. AIDS is an excellent example of the emergence of a new viral disease that occurred when changes in the ancestral simian HIV virus genome strain occurred after exposure to a new host (*Homo sapiens*). An infection that was originally zoonotic eventually adapted and established itself in human populations.

Where a micro-organism cannot be easily isolated in the laboratory, and with the advent of the molecular biology era, sequence-based methods for the identification of microbial pathogens can provide evidence of disease causation. In what follows, HIV is used as an example, in guidelines that are cited by Fredricks and Relman<sup>1</sup> for the establishment of causal relationships between a microbe and a disease:

1. A nucleic acid sequence belonging to a putative pathogen (e.g. HIV) should be present in most cases of an infectious disease (e.g. AIDS as the end-stage of HIV infection).

2. Fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts and tissues without disease (if the host is truly HIV negative, then there should be no HIV-associated nucleic acid sequences that are detectable).

3. Copy number of pathogen-associated nucleic acid sequences should decrease or become undetectable with treatment or resolution of the disease (on appropriate highly active antiretroviral therapy (HAART) regimens, successful treatment is indicated by serial HIV viral load monitoring until it reaches undetectable levels).

4. When sequence detection predates disease, or sequence number correlates with the severity of disease or pathology, the sequence-based association is more likely to be a causal relationship (e.g. HIV may be detected by HIV RNA-PCR in a patient who is completely asymptomatic and unaware of his/her infection, i.e. the presence of the sequence predates symptomatic disease, or the number of HIV copies that are detected increase with disease progression if no or an unsuccessful HAART is given).

5. When phenotypes (e.g. pathology, microbial morphology, and clinical features of HIV infection) are predicted by sequence-based phylogenetic relationships, the meaningfulness of the sequence is enhanced.

6. Tissue-sequence correlates should be sought at cellular levels (e.g. if there is tropism of HIV for cells bearing CD4 receptors it is a reasonable expectation to note the pathological effects of HIV on cells bearing such receptors).

7. Sequence-based forms of evidence should be reproducible (if testing for HIV nucleic acid sequences on the same patient's blood sample is performed by two independent researchers or sites, the results should be similar in what they reflect).

## The pathogenesis of infectious diseases

All microbes causing infectious diseases are subjected to a series of gruelling challenges, if they are to succeed. Put simply, these are a sequence of bacterial (prokaryotic) – host (usually eukaryotic) cell interactions. Firstly, pathogenic micro-organisms, e.g. bacteria, usually reside and multiply in an environment that favours their survival, replication and existence. All pathogenic (disease-causing) micro-organisms must identify a route of transmission from their reservoir (animal, human, plants, water, soil, air) to the hosts they will infect. Once the micro-organism is successfully transmitted, its first challenge is to adhere to the epithelial (mucosal) surface for which the microbe has the greatest tropism. Microbial adhesion is the first and most critical process in infection. Adhesion of a micro-organism to a mucosal cell surface or a target site can be nonspecific (as a consequence of hydrophobicity, van der Waal's forces of attraction, formation of extracellular slime) or highly specific (involving a microbe-specific host receptor interaction, e.g. the attachment of the bacterial toxin of the cholera-causing organism, *Vibrio cholerae*, to GM1 ganglioside receptors present on small intestinal epithelial cells). Adhesion may be followed by invasion of an organism which results in the passage of a bacterium through other cell populations in the epithelia (e.g. M cells in the gastrointestinal tract) or by damage of the epithelial layer. Within the host, multiplication of the organism can then occur and in so doing will evoke immune responses from the host. The host mucosal surface is constantly on guard against microbial invasion and produces an array of antimicrobial defences that microbes have to deal with. These defences include the production of antibacterial



peptides, metal-chelating compounds, destructive enzymes such as lysozyme, and mucosal and secretory IgA and other antibodies. Collectively, all of these constitute what has become the highly specialised science of mucosal immunity. For a microbe to survive, it is essential that it is armed with mechanisms to overcome or evade protective host responses. Pathology caused by microbial infection is usually due to either microbial factors (e.g. release from bacterial cells of exotoxins) or to the overproduction or hyper-responsiveness of host factors – predominantly cytokines – produced by the host as a defence against infection. The ability of a microbe to adhere, invade, evade host defences and cause tissue damage is due to its ability to produce virulence factors. These include: (i) adhesions, which promote microbial adherence to host tissues; (ii) invasins, which are responsible for tissue invasion; (iii) impedins, molecules which allow microbes to overcome host mechanisms; (iv) aggressins, factors which promote damage to host cells and tissues; and (v) immunomodulatory microbial components. Finally, as an infectious microbe survives by spreading and amplifying itself from host to host, it must be able to exit from the host and encounter another uninfected host to start the infectious cycle anew. Alternatively, the microbe must return to, and persist in, its reservoir.

Let us divert the discussion for a moment from bacteria to viruses and consider the applicability of the above concepts to Marburg virus. Like its closely related species, Ebola, it belongs to the family Filoviridae. The genus *Filovirus* is separated into two distinct species, Marburg and Ebola, which differ significantly in their glycoprotein (GP) genes. The divergence between the two species of *Filovirus* implies that both have a common ancient ancestral origin and they have slowly (perhaps over a period of five millennia) co-evolved with their as yet unconfirmed natural hosts. The reservoir of both Marburg and Ebola viruses, though speculated and much debated, remains unknown. Marburg virus is probably a zoonotic pathogen but when primates including man are infected with this microbe, it can cause devastating disease as a consequence of its formidable virulence. The agent is transmitted to a new host as a consequence of the latter coming into contact with virus-contaminated body fluids that have been excreted by the initial host infected with this virus.

The pathogenetic sequence of infection, as described by and summarised from Bray,<sup>8</sup> involves the following steps:

1. Initial infection of macrophages, dendritic cells and other cells of the mononuclear phagocytic system (MPS), probably in regional lymph nodes occurs. The cell surface receptor of filoviruses has not been identified but electron microscopy studies suggest that filoviruses bind to a broad range of cells and enter the cell through endocytosis. Intracellular viral replication is accompanied by cytoplasmic vesiculation and mitochondrial swelling, breakdown of organelles, and terminal cytoplasmic rarification.

2. Replication of virus is accompanied by suppression of

interferon (INF)- $\alpha/\beta$  which allows rapid local and systemic dissemination.

3. MPS cells that migrate to other tissues release free virions into the lymphatics or bloodstream, resulting in systemic dissemination, infecting fixed tissue macrophages in the liver, spleen and other tissues around the body.

4. Virions released from the above-mentioned cells infect, among others, hepatocytes, adrenal cortical cells, fibroblasts, and endothelial cells in adjacent blood vessels.

5. Infected macrophages, once activated, release large quantities of cytokines and chemokines (tumour necrosis factor (TNF)- $\alpha$ , monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1 $\alpha$ , etc.).

6. Increased permeability of the duodenum, leakage of macromolecules, expression of endothelial cell surface adhesion and procoagulant molecules, together with tissue destruction, result in exposure of underlying collagen and release of tissue factor. This leads to disseminated intravascular coagulopathy (DIC), platelet dysfunction and progressive hepatic failure.

7. Massive cytolysis, fluid shifts, effects of cytokines, interstitial haemorrhage and tissue ischaemia (from diffuse obstruction of capillary blood flow by masses of virions and microthrombi), all contribute to mortality.

Marburg virus is associated with an exceptionally high mortality (80 - 90%) of all infected and microbiologically confirmed clinical cases. During the course of clinical illness, contact with blood and body fluids from the infected host is the main mechanism whereby the virus leaves its first host, and infects another anew. During interepidemic periods, the virus returns back to its elusive reservoir/s.

## Viewing infection through a biofilm

The concept of microbes, e.g. bacteria, living in biofilms, was first described by the great Dutch microscopist, Antoni van Leeuwenhoek, in 1663. Using a single-lens microscope he described 'animalcules' in the mouths and on teeth of humans. Quoted in Henderson *et al.*,<sup>9</sup> van Leeuwenhoek wrote: '... But what if I should tell such people in future that there are more animals in the scum on the teeth (dental plaque) in a man's mouth, than there are men in the whole kingdom?'

It could be argued that for the last 50 years we have devoted most of our efforts in studying the minor bacterial phenotype (planktonic cells), ignoring the major phenotype (biofilm-embedded sessile cells) predominant in natural and pathogenic ecosystems. A biofilm is, in essence, an assemblage of microbial cells, irreversibly associated with a surface, and enclosed in a matrix of mainly, but not exclusively, polysaccharide material. Thus the long-held view that bacteria are a simple, primitive, version of the evolutionarily superior eukaryotic cell, acting as a unicellular automaton, is no longer correct. As our understanding of bacteria accumulates, it is now appreciated that they are complex, craftily adapted and



highly adaptable micro-organisms which can respond to their environment in myriad, varied ways. The concept of a consortium of cells living within a biofilm matrix is indeed a form of multicellularity, giving bacteria huge survival advantages that eukaryotic, multicellular organisms enjoy. Biofilm multicellularity results in better bacterial defences against the host. Nutrient depletion in the sessile cells that are most deeply embedded in a biofilm creates zones of altered metabolic activity. A distinction needs to be made between sessile (inner) and planktonic (outer) biofilm cells. Sessile cells are small, tightly adherent and relatively metabolically inactive cells that are found deeply embedded at the bottom of a biofilm and that adhere tightly on a variety of surfaces: In natural aquatic systems these could be rocks in a river, in industrial systems these could be water tanks or pipes and in the medical setting these surfaces might include medical devices (e.g. cardiac pacemakers, prostheses, central venous catheters, etc.) and living tissues (e.g. the lungs in cystic fibrosis, gallstones in cholecystitis, prostate in prostatitis, etc.). As the outer (planktonic) cells of a biofilm absorb damage (including the onslaught from antibiotics), this allows inner layers of biofilm cells to buy more time to initiate a stress response. A higher number of 'persister' cells are present in the biofilm. Bacterial biofilms confer physiological adaptive antimicrobial resistance to the bacterial cells contained therein: This may be due to one or a combination of the following factors: (i) antimicrobial neutralisation; (ii) nutrient-limited physiology; and (iii) penetration failure. Furthermore, the metabolic inactivity of sessile bacterial cells makes it difficult for antibacterial agents to function effectively. Although biofilms can be dealt with using antibiotics, 1 000 - 1 500 times higher doses are required to kill biofilm versus planktonic cells. In much the same way as multicellular organisms are dependent on intercellular communication or signalling for their survival, it is now appreciated and proven that bacteria do signal to each other. The term 'quorum sensing' refers to the ability of bacteria within a population, e.g. a biofilm, to determine their cell density and consequently, as required, switch on (or off) the expression of particular genes or operons (sets of genes) that favour their survival. It could well be that when a bacterial population reaches a critical cell density (i.e. it is quorate) census taking by bacteria is a virulence mechanism to allow them to increase in numbers, without switching on virulence genes that might alert the host to their presence. Quorum sensing is not the only example of the complex interactions bacteria have with their environment. Prokaryotic-eukaryotic communication also occurs: bacteria can respond to signals from eukaryotic host cells as well react to direct cell-to-cell contact with host cells. Bacteria are responsive to a wide array of mammalian mediator molecules such as serotonin, catecholamines, insulin and cytokines including interleukin (IL)-1, IL-2, TNF, and transforming growth factor (TGF). Bacteria can themselves produce a variety of immunoregulatory molecules that result in the release of cytokines that are not only key cell-to-cell regulatory

molecules in multicellular organisms, but also control immune responses (both innate and acquired) in vertebrates including humans. Macrophages and other cells of the host's immune system attempt to penetrate the biofilm but become trapped. In so doing these very same cells release cytokines that cause damage in the host.

## The renewed understanding of infectious diseases – the molecular biology era

Molecular biology techniques have enabled our understanding of infection to progress far beyond the boundaries that we thought we had reached. Understanding cell-to-cell interactions at the level of molecules, having techniques that enable us to amplify genes unique to specific microbial cells, our ability to perform DNA fingerprinting of organisms to establish their clonality or relatedness, the ability of characterising entire genomes of microbes as well as their specific virulence factors all have radically transformed the pathogenetic understanding, molecular epidemiology, diagnosis, and approaches to treatment of infectious diseases.

## Conclusion

Microbes are not 'just microbes'. As we are only beginning to grasp the extraordinary nature of what we previously considered 'simple' organisms, we are humbled by the microbial world. The sometimes uneasy relationship between man and microbe, that may lead to devastating disease and death of millions of humans each year, is a sober reminder that mankind is in charge of its destiny only to a limited degree. Despite advances in our knowledge of infectious diseases and the development of an array of novel therapeutic strategies, the ability of the microbial world to adapt, develop new strategies for survival, develop new virulence traits, express novel mechanisms for antimicrobial resistance is a poignant reminder that man is just *Homo sapiens*, one of the many forms of life on our earth. We are so heavily colonised by microbes that 90% of the human body, purely on the basis of cell numbers, consists of bacteria. By understanding this concept, we should reflect on the miraculous, diverse and complex nature of our universe.

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